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Angiogenesis Related Markers In Non-Small Cell Lung Cancer

BY

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Abstract

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This thesis investigated the predictive and the prognostic powers of angiogenesis related markers in both operable and inoperable non-small cell lung cancer (NSCLC) patients.

In the first and second study, we investigated the serological fractions of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in 2 cohorts of patients with either operable or inoperable NSCLC.

Regarding operable NSCLC, we demonstrated significant correlations between VEGF and tumour volume and overall survival. Regarding bFGF, significant correlations with recurrent disease and survival were demonstrated. VEGF and bFGF correlated to each other and with platelet counts. In multivariate analysis, bFGF proved to be a significantly independent prognostic factor.

Regarding inoperable NSCLC, we demonstrated that patients with elevated bFGF levels before any treatment and during chemotherapy had a significantly poorer survival. During chemotherapy, each rise of one unit of bFGF (ng/L) corresponded to a 4 times increased risk of death. Regarding VEGF, elevated levels after radiotherapy corresponded with better survival. All prognostic information demonstrated in this study concerned patients with a, co-sampled, normal platelet count.

In the third study, three putative markers, HER-2, EGFR and COX-2, suitable for targeted therapies in resected NSCLC were investigated in a panel of 53 tumours and further investigated for a possible correlation with microvessel density. We demonstrated that HER-2 and COX-2 were mainly expressed in adenocarcinomas, whereas EGFR was only expressed in squamous cell carcinomas. COX-2 showed a trend towards a correlation with microvesssel density. The expression profile, HER-2+/EGFR-, was significantly correlated to poorer survival.

In the fourth study, a predictive model for recurrences consisting of p53, CD34 and CD105, and circulating serum fractions of VEGF and bFGF, was investigated. The two endothelial markers correlated with each other. CD105 expression correlated with p53 expression. No other significant correlations between markers could be demonstrated. A significant correlation between p53 overexpression and recurrent disease was demonstrated. The mutational status could not confirm the immunohistochemical correlation between p53 and recurrences.

In conclusion, the present thesis demonstrates that the angiogenic factors VEGF and bFGF analysed in sera have both predictive and prognostic information when measured in operable and inoperable NSCLC. Since HER-2 is overexpressed in NSCLC and linked with prognostic information, this marker might be a suitable target for therapy in NSCLC. Furthermore, in patients with operable NSCLC, p53 expression status was linked with recurrent disease and mean MVD.

Keywords: Lung cancer, NSCLC, Therapy, Angiogenesis, VEGF, bFGF, Microvessel density, Survival

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Original papers

The present thesis is based on the following original papers, which are referred to in the text by their as Roman numerals:

I. Elevated preoperative serum levels of angiogenic cytokines correlate to larger primary tumours and poorer survival in non-small cell lung cancer patients. Published, Lung Cancer 2002:37(1):57-63.

II. Serum VEGF and bFGF adds prognostic information in patients with normal platelet counts when sampled before, during and after treatment for locally advanced non-small cell lung cancer. In Press: In Lung Cancer.

III. HER-2, EGFR, COX-2 expression status correlated to microvessel density and survival in resected non-small cell lung cancers. In Press: Acta Oncologica.

IV. Endothelial markers and circulating angiogenic factors and p53 as markers for recurrence in surgically resected non-small cell lung cancer patients. Submitted: Neoplasia.

Contents

Abbreviations

2D-PAGE	2 Dimensional Polyacrylamide-Gel-Electrophoresis
COX-2	Cyclooxygenase 2
ECM	Extracellular matrix
EDTA	Etylendiamintetraacetat
EGFR/HER-1	Epidermal growth factor receptor/ Human epidermal growth factor receptor-1
FGF-1/aFGF	acidic Fibroblast Growth Factor
FGF-2/bFGF	basic Fibroblast Growth Factor
Flk-1	Fetal liver kinase-1
Flt-1	Fms-like tyrosine kinase-1
HER-2	Human epidermal growth factor receptor 2
HGF/SF	HepatocyteGrowth Factor/Scatter Factor
KDR	Kinase Domain Receptor
MVD	Microvessel density
NSCLC	Non-Small Cell Lung Cancer
PBS	Phosphate Buffered Saline
PD-ECGF/TP	Platelet Derived Endothelial Cell Growth Factor/ Thymidine Phosphorylase
PDGF	Platelet Derived Growth Factor
PECAM	Plateletderived Endothelial Cell Adhesion Molecule
PKC	Proteinkinase C
PlGF	Placental Growth Factor
PNET	Primitive neuroectodermal tumour
SCLC	Small Cell Lung Cancer
SELDI-TOF	Surface Enhanced Laser Disorption Ionisation- Time of Flight
SH2	Src-homology 2
TGF-α	Transforming Growth Factor alpha
TGF-β	Transforming Growth Factor beta
TNF- α	Tumour Necrosis Factor alpha
VEGF	Vascular Endothelial Growth Factor
VEGFR-1	Vascular Endothelial Growth Factor Receptor-1
VEGFR-2	Vascular Endothelial Growth Factor Receptor-2
vWF	von Willebrand factor

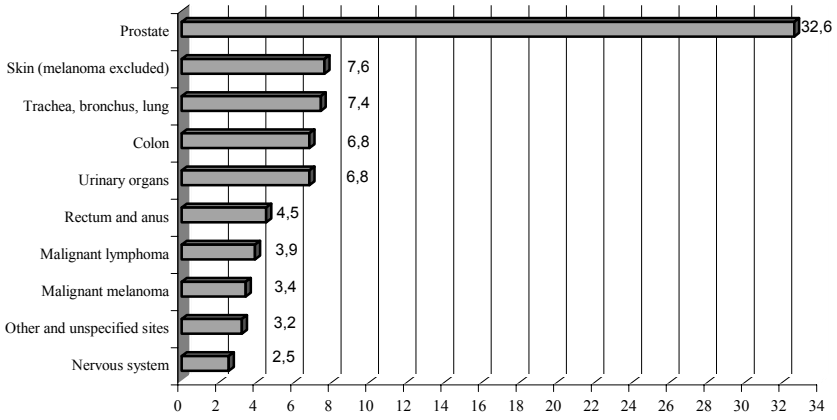
Introduction

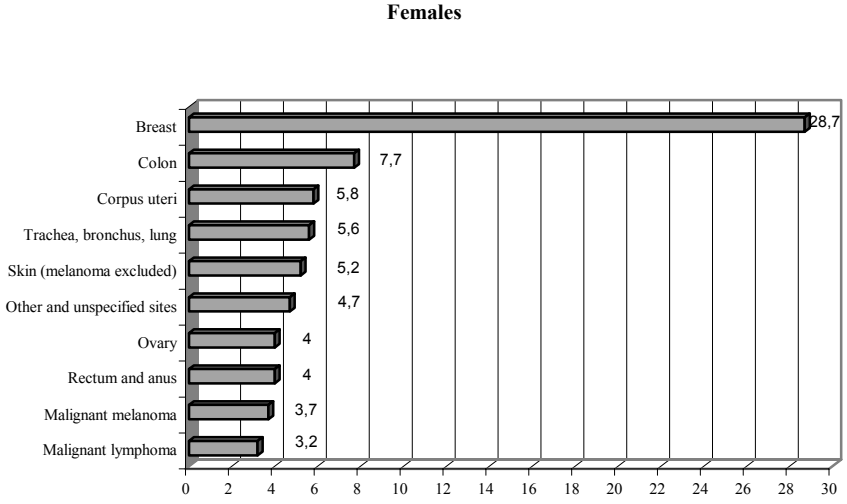
Lung cancer

Lung cancer was a rare disease until the beginning of the twentieth century, but since then it has become the most common malignancy worldwide, in terms of both incidence and mortality [1].

In Sweden, total cancer incidence in year 2000 was 45 482, among them being 38 907 persons that were diagnosed for first time. From these data, an incidence rate per 100 000 in males with the first primaries can be calculated to 511.9 and the corresponding incidence rate for females to 389.9. Lung cancer in Sweden is the third most common cancer in males, 7.4 % of all tumours, and the fourth most common in females, 5.6 % of all cancers (figs. 1 & 2).

Males





Figs 1 & 2. *The ten most frequent specified cancer sites, by sex. Cancer incidence in Sweden 2000. National Board of Health and Welfare, Cancer Statistics, Sweden*

These data correspond to 2 846 newly diagnosed primary lung cancers each year. In women, the incidence rate is still rising, with an average increase of 2.6 % per year, whereas the previously noted increase in men has now turned to a decrease of 1.6 % annually [2]. Tobacco smoking is by far the most important risk factor for lung cancer, yet other agents, for example, asbestos and radon, have been showed to be possible risk factors. Among the 4000 identified chemicals in cigarette smoke, more than 60 are established carcinogens. The strongest carcinogens are polycyclic aromatic hydrocarbons (PAHs), *N*-nitrosamines, and aromatic amines. In relation to human lung cancer, arguably the most important carcinogens are the PAHs benzo[*a*]pyrene and the tobacco specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (also known as NNK) [3]. These compounds occur in small quantities, typically about 5–200 ng per cigarette. For tobacco smoking there is a clear linear dose-response relationship with no threshold

dose, e.g. even 1 cigarette per day leads to a higher risk of contracting lung cancer.

Lung cancers are divided into two major groups depending on their morphological appearance, as summarised in Table I. The two groups are small cell lung cancer (SCLC) with 15-20% of the lung cancer cases, and non-small cell lung cancer (NSCLC), which accounts for approximately 75-80% of the lung cancer cases. NSCLC can be further divided into adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, large cell carcinoma and bronchioalveolar carcinoma [4]. The subtyping of NSCLC depends on morphology, however the routine use of immunohistochemical stainings for the verification of the diagnosis is increasing, Table I.

Table I. Morphological characteristics and differential diagnosis in NSCLC and SCLC, summarised and adapted from Travis et al [4]. Shortened version. Italics within brackets indicate differential diagnosis

1.3.1 Squamous carcinoma (Sq.)
Keratin pearls (high diff), epithelioid sheets (poor diff), *[SCLC]*
Intercellular bridges, *[Ad.sq.]*
Eosinophilic or clear cytoplasm, *[Thymoma]*
Coarse chromatin and dense, often shrunken nucleus, *[Met. sq.]*
Geographic necrosis common
Uncommonly clear cell, papillary, small cell, or basaloid variants
1.3.2 Small cell carcinoma
Small cells (less than three mature lymphocyte diameters), scant cytoplasm, *[SCLC, combined]*
Frequent mitoses, *[Non-small cell lung carcinoma]*
Finely nuclear chromatin ("salt and pepper" pattern), *[Lymphoma]*
Nuclear moulding, *[PNET]*
Crush artefact frequently prominent, *[Synovial sarcoma]*
1.3.3 Adenocarcinoma (Ad.)
Destructive growth pattern with alveolar destruction, *[BAC]*
Fibrous stroma (may be associated with apical scar, "scar carcinoma")
Gland-like structures, tubules (acinar tumours), *[SCLC]*
Papillary structures (papillary tumours), *[Mesothelioma]*
Signet ring cells (more common in metastasis), *[Met. ad. & melanoma]*
Mucin (documented in mucin and PAS stains)
Uncommonly "fetal," mucinous, or clear cell variants
Prominent nuclear membranes, large nucleoli
1.3.3.3 Ad., Bronchioloalveolar carcinoma (BAC)
Cuboid or columnar cells line alveoli with minimal architectural destruction, *[Ad., acinar type]*
Minimal stromal response except for central collapse, *[Mesothelioma]*
Mucinous (goblet cell) or nonmucinous (Clara/type II cell), *[Met. ad.]*
Prominent nuclear membranes, large nucleoli, *[Type II cell hyperplasia]*
Multifocal, *[Atypical alveolar hyperplasia]*
1.3.4 Large cell carcinoma, undifferentiated (LCLC)
Large cells without glands, keratin pearls, intercellular bridges, *[SCLC]*
Moderate cytoplasm, *[Sq.]*
High mitotic rate, *[Ad.]*
Coarse chromatin, prominent nucleoli, *[Ad.sq]*
Neuroendocrine, basaloid, lymphoepithelial, clear cell, rhabdoid variants
1.3.5 Adenosquamous carcinoma (Ad.sq.)
Glands, mucin production (>10%) and squamous differentiation (>10%)
[Sq.], [Ad.], [Mesothelioma], [Met. ad.], [Mucoepidermoid carcinoma]

The golden standard regarding treatment differs between the two major groups. This thesis focuses on NSCLC, and consequently SCLC treatment is only briefly discussed.

SCLC treatment

When diagnosed, SCLC is divided into limited or extensive disease. The term limited disease is used when the tumour is confined within the thorax cavity and thus, when the tumour has spread outside the thorax cavity, the term extensive disease is used. For limited disease there is a benefit of combining chemotherapy with radiotherapy, and patients responding with a complete remission should receive prophylactic cranial irradiation in order to reduce the risk of brain metastases [5]. In Sweden, the chemotherapy treatment of choice for SCLC is four to six courses of combined Etoposide and platinum-containing regimes, but recent data indicate that Irinotecan might be more efficient than Etoposide [6]. When radiotherapy is planned, the aim towards a total dose of 45-50 Gy to the primary tumour is preferred, and when prophylactic brain irradiation is planned, the goal is a total dose of 30-36 Gy to the whole brain [7]. In extensive disease, chemotherapy as described earlier is offered, whereas radiotherapy only has a place in the palliative situation [7]. Studies presented with the above approach indicate a benefit in terms of survival, yet long-term survivors are still between 5-10% [8].

NSCLC treatment

For NSCLC, the treatment depends on stage of the tumour. Staging classification is mainly based on tumour size, relation to the main bronchus or thoracic wall, node involvement and metastases (TNM), Tables II and III. Staging is based on pulmonary X-rays, CT-scans of the thorax and upper abdomen, bronchoscopy and, in elective cases, mediastinoscopy. The classification follows the recommendations from UICC, Union

Internationale Contre le Cancer [9]. Potentially resectable early stage NSCLC (stage I-IIIA T3) patients are offered operation, if manageable regarding medical conditions. T1 and T2 tumours, after surgical resection, have a 5-year survival rate of 70-50% [10]. Nevertheless, less than half of the patients who have undergone complete resection are cured [11]. So, recent trials have addressed perspectives in both improving distant failures and local control.

Table II. TNM descriptors, summarised from Mountain CF [9].

Primary tumour (T)	
TX	Primary tumours cannot be assessed, or proven by the presence of malignant cells in sputum or bronchial washings but not visualised by imaging or bronchoscopy.
T0	No evidence of primary tumour
Tis	Carcinoma in situ
T1	Tumour =<3 cm in greatest dimension, surrounded by lung or visceral pleura. No bronchoscopic evidence of invasion proximal than the lobar bronchus (eg. not in the main bronchus)
T2	Any of following features: tumour>3 cm, involves main bronchus, =>2 cm from carina, invades visceral pleura, associated with atelectasis or obstructive pneumonitis that extends to the hilar region but does not involve the entire lung
T3	Tumours of any size that directly invade any of the following: chest wall, diaphragm, mediastinal pleura, parietal pericardium; or tumour in main bronchus <2 cm distal to carina but without involvement of the carina; or associated atelectasis or obstructive pneumonitis involving the entire lung
T4	Tumours of any size that invade any of the following: mediastinum, heart, great vessels, trachea, oesophagus, vertebral body, carina; or tumours with malignant pleural effusion; or satellite tumours within the same lobe as the primary lung tumour

Table II. Continued

Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed.
N0	No regional lymph nodes
N1	Metastases to ipsilateral peribronchial or hilar lymph nodes, and intrapulmonary lymph nodes involved by direct extension of the primary tumour
N2	Metastases to ipsilateral mediastinal and/or subcarinal lymph nodes
N3	Metastases to contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene or supra-clavicular lymph nodes
Distant metastases (M)	
MX	Present of distant metastases cannot be assessed
M0	No distant metastases
M1	Distant metastases present

Table III. Stage grouping among TNM subsets, summarised from Mountain CF [9]

TNM-subsets	**Stage**
Carcinoma in situ	0
T1N0M0	Ia
T2N0M0	Ib
T1N1M0	IIa
T2N1M0, T3N0M0	IIb
T3N1M0, T1N2M0, T2N2M0, T3N2M0	IIIa
T4N0M0, T4N1M0, T4N2M0, T4N3M0	IIIb
T1N3M0, T2N3M0, T3N3M0	
Any T Any N M1	IV

Chemotherapy NSCLC

In the adjuvant setting (surgery vs. surgery plus chemotherapy), data from a meta-analysis including 4357 patients indicated a disadvantageous effect on survival when long-term alkylating agents were used [12]. A 15% increased risk of death was calculated, whereby the 5-year survival was reduced from 50% to 45% in absolute numbers. But when cisplatinum-regimes were administered in the adjuvant setting a survival benefit was shown, as a reduction of 13% in the risk of death was demonstrated. This suggests an absolute benefit from cisplatinum-containing chemotherapy of 5% at 5 years, improving survival from 50 to 55% [12]. A more recent publication regarding adjuvant therapy is EST 35590. This study, including stage II and IIIa patients, investigated the adjuvant use of chemotherapy plus radiation therapy vs. postoperative radiation alone, could not demonstrate a survival benefit in the combination arm [13]. The most recent adjuvant study, IALT, was presented as an abstract at ASCO 2003 [14]. This study randomised patients either to surgery plus chemotherapy or surgery alone. The collaboration group included 1867 patients with stage I-III NSCLC, 932 in the control arm and 935 patients in the experimental arm. They demonstrated that patients receiving adjuvant chemotherapy had a survival benefit of 4.1% in absolute numbers in the experimental arm compared with the control arm. Other interesting adjuvant trials are ongoing, at least two more European and one North-American, and hopefully will the solution to the use of adjuvant CT in NSCLC will be closer after these studies have been presented [15].

In the neo-adjuvant approach (surgery vs. preoperative chemotherapy plus surgery), Roth et al. [16] and Rosell et al. [17] randomised patients between treatment groups and both authors reported a median survival advantage in the preoperative chemotherapy plus surgery group, 22 months compared with 10 months, respectively. However, due to small sample size (30 patients in respective arms, both studies) the magnitude of the treatment

benefit remains unsolved. In late 90s, Rosell et al. [18] launched an interesting study comparing neoadjuvant paclitaxel plus carboplatin, or surgery alone, or post-operative chemotherapy with the same regimen (NATCH study). This study is ongoing, and preliminary data point in the same direction as the other preresectional chemotherapy studies, which is towards a better response rate in the group having preoperative chemotherapy [18].

Regarding locally advanced lung cancer, the previous standard treatment was radiation therapy. Now, convincing evidence has been presented on the beneficial effect of combining radiation and chemotherapy [12, 19]. The meta analysis demonstrated a significant overall benefit of combining radiation and chemotherapy, the hazard ratio of 0.90 (P=0.006), or 10% reduction in the risk of death; corresponding to absolute benefits of 3% at 2 years and 2% at 5 years [12]. When subgroup analysis was done, cisplatinum-containing therapies demonstrated the strongest evidence for an effect in favour of chemotherapy, with a hazard ratio of 0.87 or a 13% reduction in the risk of death, which corresponds to absolute benefits of 4% at 2 years and 2% at 5 years (P=0.005) [12].

In advanced disease, regarding supportive care vs. supportive care plus chemotherapy, the meta-analysis consisting of 1190 patients and 1144 deaths, demonstrating a detrimental effect of chemotherapy with a 26% increase in the relative risk of death when using long-term alkylating agents. But, the confidence intervals were wide (0.96 to 1.66) and the result did not reach conventional levels of significance (P=0.095). When cisplatinum-based trials were analysed, data demonstrated a clear benefit of chemotherapy, with a hazard ratio of 0.73 (P<0.0001) or a 27% reduction in the risk of death [12], which corresponds to an improved median survival from 4 months to 5.5 months. Data are accumulating regarding the additive beneficial effects with the combined use of platinum and newer agents, such as gemcitabine, taxanes, vinorelbine and topoisomerase-1 inhibitors, in

advanced NSCLC [20-27]. A recent ECOG1594 study compared four chemotherapy regimens, cisplatin plus paclitaxel vs. cisplatin plus gemcitabine vs. cisplatin plus docetaxel vs. carboplatin plus paclitaxel. The study included 1207 patients whereas 1155 patients were eligible for analysis. There were no significant differences in survival between the regimens, but the regimen of carboplatin and paclitaxel had a lower rate of toxic effects than the other regimens [28].

To date, until more data are at hand, it seems that the use of platinum-based chemotherapy, preferably combined with one of the newer agents, is the best choice when planning first line treatment for advanced NSCLC [29]. The benefit of second line treatment with docetaxel for lung cancer, previously treated with platinum-containing therapy, has been presented but only one study has compared treatment with best supportive care [30], whilst the other study investigated three different chemotherapy regimes with three active agents [31]. Docetaxel (75 mg/m²) compared with BSC, gave an extra 3 months survival, median 7.6 months vs. 4.6 months, and a one-year survival of 37 % in the docetaxel group compared with 19% in the BSC group [30]. The study comparing three active agents, docetaxel 100mg/m2 or docetaxel 75 mg/m2 vs. an control arm of either vinorelbine 30 mg/m2 or ifosfamide 2 g/m2, demonstrated a survival benefit and better global quality of life in the docetaxel 75 mg/m2 group [31].

Radiotherapy NSCLC

Outcome of radiotherapy for NSCLC depends on different aspects, such as, for example, patient characteristics (e.g. age, gender and performance status), target volume/definition, dose per fraction, total dose, treatment length and combined chemotherapy. In a study performed by the Radiation Therapy Oncology Group trial for lung cancer (RTOG), three different radiation doses of 40, 50 and 60 Gy, delivered with daily 2 Gy fractions

were evaluated in terms of percentages of local control and survival [32]. They found that 3-year survival rates were 6% after 40 Gy, 10 % after 50 Gy and 15 % after 60 Gy. Furthermore, 60 Gy induced a better local control than 40 Gy and, thus, the probability of controlling a tumour increased with the higher doses. Radical radiotherapy, with a total dose above 60 Gy, alone can accomplish a 5-year survival year in about 30-50% of the patients with T1 or T2 tumours with no signs of lymph node involvement not eligible for curative surgery, depending both on stage and response to therapy [33, 34]. The use of more advanced conformal radiotherapy with more accurate positioning and with a stereotactic approach, using differing fixation techniques, with higher total doses, the local control rates and crude and disease-specific survivals increase. [35-38]. Local control rates above 80% [37, 38] and 3-year crude and disease specific survivals of 66% and 88%, respectively [38], are described. Regarding patients who underwent surgery for early operable NSCLC, the most recent update from PORT Postoperative Meta-analysist Trialists Group regarding postoperative radiotherapy in early NSCLC, involving 2128 patients from 9 trials, demonstrates that adding postoperative radiation therapy for patients with completely resected NSCLC severely increased the relative risk of death by 21%. This is equivalent to an overall reduction in survival from 55% to 48% at 2 years. Further exploratory analysis by stage and by nodal status suggested that this detrimental effect was most pronounced for earlier stage patients and those with lower nodal status. For stage III and N2 patients no clear evidence of a difference between surgery or surgery plus radiation therapy [39] could be demonstrated. However, criticism of the results of this study has been raised since many of the trials investigated in this study used outdated radiotherapy techniques, which might have caused increased risk of lethal toxicity, e.g. pneumonitis [40, 41].

In locally advanced NSCLC, there is enough evidence to indicate that radiotherapy combined with chemotherapy improves survival, as described in the section on *Chemotherapy NSCLC*, so future research on radiotherapy with primary endpoint survival probably will include chemotherapy, perhaps as induction therapy or in different concomitant settings. In the palliative setting, radiotherapy has an indisputable place as a symptom reliever. Current recommendations concerning radiotherapy in Sweden are demonstrated in Table IV.

Table IV. Radiation treatment for patients with NSCLC and SCLC

Stage	**Recommendation**
I, II , IIB	Patients that are medically not operable, curative intended radiation treatment =>60 Gy
Tumours engaging the thoracic wall (T3, N0/N1, M0)	Incomplete thoracic surgery, radiation treatment > 60 Gy in combination with chemotherapy
	Radiation treatment =>60 Gy, often in combination with chemotherapy.
IIIA	Mostly combined chemoradiation. For some patients, surgery might be of supplementary value.
IIIB	Radiation treatment >60 Gy, often in combination with chemotherapy
IV	Palliative radiotherapy
Vena cava superior syndrome	Mainly radiation treatment but in selected cases, other local treatments

Future treatments NSCLC

Although recent advances in the treatment modalities regarding NSCLC in total, there is only a modest improvement in overall survival. The use of conventional chemotherapy seems to have, at least in locally advanced

NSCLC, reached its plateau. Now, even though in a small subpopulation of NSCLC patients, we are awaiting further confirmatory adjuvant chemotherapy trials to see if we have moved the overall survival even further up the slope, as could be demonstrated in the IALT study.

Intuitively, this approach will probably lead to a small but significant prolongation of the overall survival, as earlier described [14], but the use of newer agents focusing on different aspects on the tumour cells molecular biology will sooner or later lead the way to substantial clinical breakthrough. The treatment paradigm towards the goal of total eradication of tumour cells will be challenged as we probably will focus on life-long therapies, inducing for instance tumour dormancy, instead. Recent advances and increasing understanding of tumour biology have rendered many new investigational anticancer drugs [42] with different proposed mechanisms of actions. Today, there are probably more drugs in clinical tests than the reported 209 drugs in the overview by Nygren et al [42]. Data from randomised clinical controlled trials were available for 28 out of these 209 drugs, and the drugs could be classified into different groups depending on mechanistic actions, as for example targeting tumour antigens or directly the genome, as for example in the antisense treatments.

In the present thesis, one of these targets, the angiogenic process, is highlighted indirectly through studies on different relationships between angiogenic cytokines, signal transduction pathways and other interesting molecular aberrations and their implications in the clinical setting.

Angiogenesis

Vessel growth can be divided into vasculogenesis and angiogenesis. Vasculogenesis is the initiation of vessels in the embryogenesis, where blood islands migrate down into organs and become vessels at site [43]. The discovery of endothelial cell precursors in the adult peripheral blood, which are able to differentiate as endothelial cells in vitro and to participate in angiogenesis in vivo [44], have demonstrated the existence of postnatal vasculogenesis [45]. Angiogenesis, on the other hand, is the initiation of endothelial cell growth and forming of new vessels from the existing vascular bed [46]. This is a normal process under tight regulatory control, and present under normal conditions in, for example, wound healing, the menstruation cycle and ovulation [43]. The tight regulatory control of angiogenesis has been postulated to be balanced between angiogenic stimulators and angiogenic inhibitors [47]. Recent publications argue over whether or not there is a definite single step event as the "angiogenic switch", as compelling evidence accumulates regarding the actions of oncogenes and tumour suppressor genes responsible for the deregulation of angiogenic properties of tumour cells in a continuum, or "angiogenic progression", as Rak et al. [48] propose. However, there is strong evidence that tumours are dependent on angiogenesis [49] and it is generally believed that tumour progression beyond a volume of approximately 1-2 cubic mm requires angiogenesis. In tumours, switching/-progressing to a more angiogenic phenotype, the net balance is in favour of angiogenic stimulators, whereas in normal settings and benign tumours the endothelial quiescence is achieved by the dominance of negative regulators [47]. In response to an angiogenic stimulus, a sequence of events occurs that culminates in the formation of new vessels. These steps in sequential order are:

1) Retraction of pericytes from the abluminal surface of the capillary.
2) Release of proteases such as urokinase, a plasminogen activator, from endothelial cells.
3) Degradation of extracellular-matrix.
4) Migration of endothelial cells and possibly proliferation.
5) Alignment of the migrating cells into tube-like formations.
6) Anastomosis of these tube-like formations and initiation of blood flow.

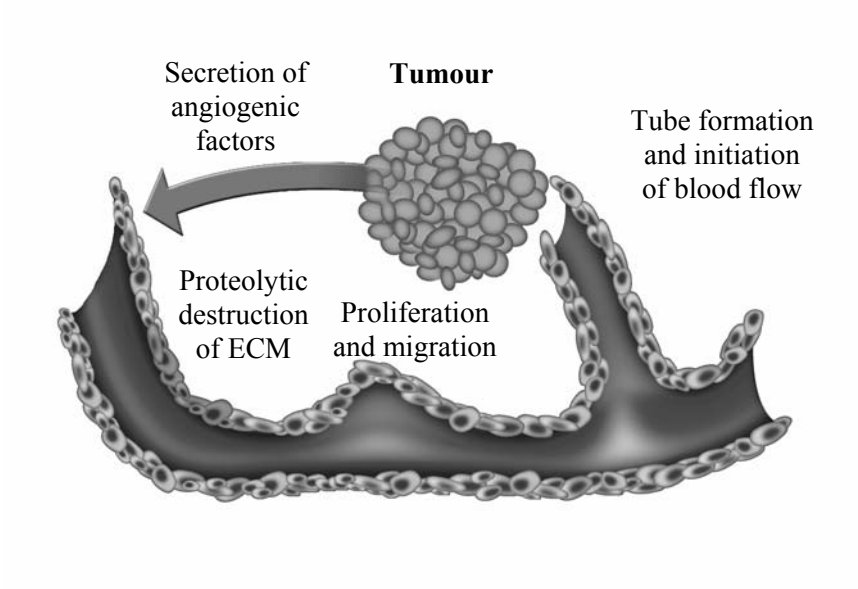


Fig. 3. The cascade of events in the angiogenic process, simplified.

thus, angiogenesis requires at least three steps: degradation of ECM, chemotaxis towards an angiogenic stimulus, and proliferation of endothelial cells and presents a complex multifactor process involving both tumour and tumour surrounding stroma [46, 50].

Angiogenesis can be studied with many different in vivo assays, some being quantitative and others being qualitative. Examples of classic in vivo assays for angiogenesis include the hamster cheek pouch [51], the chick

chorioallantoic membrane (CAM) [52] and iris and avascular cornea of the rodent eye [53]. Newer in vivo assays analysing angiogenesis apply subcutaneous implantation of various substances, e.g.s the sponge implant models [54], Matrigel plugs [55], and alginate-tumour pellets [56] or combinations of either of these model systems such as the Matrigel/Sponge model [57]. These in vivo assays are useful in elucidating the mechanism of action of a variety of angiogenic factors and angiogenesis inhibitors, and will be useful in testing novel anti-angiogenic agents before clinical trials. However, all models carry their own built-in disadvantages and should be considered before use [58].

In the clinical setting, analysing the intra-tumoural microvessel density (MVD) of solid tumours with the technique first described by Weidner et al. [59], with high magnification light microscopy on "hot-spot" areas, highlighted with specific markers for endothelial cells, like vWF, CD31 (platelet derived endothelial cell adhesion molecule/PECAM), or CD34, seems to provide prognostic information in solid tumours, such as breast [60], prostate [61], colorectal [62], laryngeal [63] and lung carcinomas [64, 65]. Regarding primary lung cancer, prognostic information seems to have been encountered, regardless of which of the above mentioned endothelial markers that have been used [66]. Today, the most widely used endothelial marker is CD34, where Mert et al.[66] demonstrated a hazard ratio of 1.99 in a review of the publications based on the prognostic impact of MVD in NSCLC [66], fig. 4.

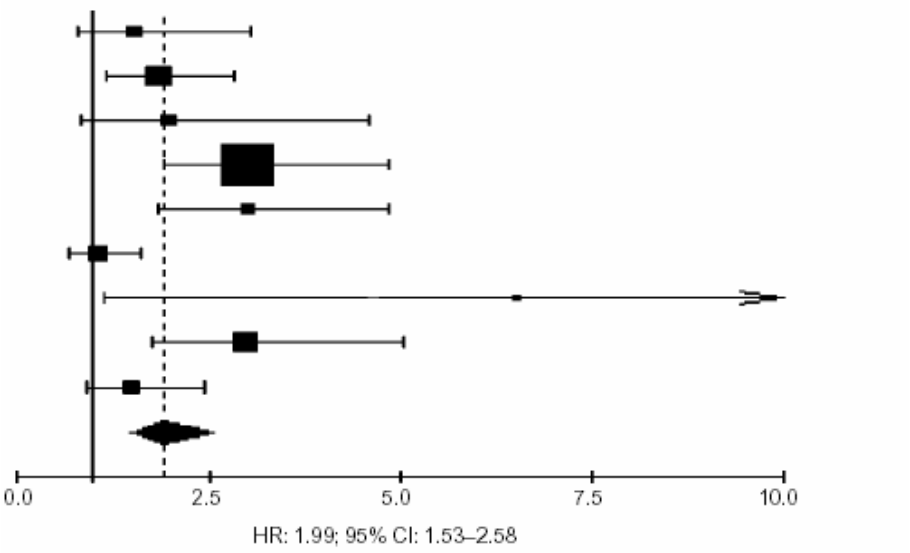


Fig. 4. Results from the meta-analysis on studies using CD34 as a marker for endothelial cells. Adapted from Mert et al. [66]

Nevertheless, several studies have failed to find that MVD is a significant prognostic factor [67-69]. Recent studies suggest the superiority of CD105 over CD34 as a marker for angiogenesis [60, 70, 71]. From the group of Wang & Kumar, CD105 has been demonstrated to be upregulated in irradiated HUVEC cells compared with normal cells [72] and to be overexpressed in breast tumour blood vessels compared with vessels of most normal tissues [73]. Furthermore, they demonstrated that in penumbra of infarcts in stroke patients, CD105 was overexpressed compared with endothelial cells of normal brain tissue [74]. One probable reason for the conflicting results may be the reactivity of these anti-endothelial cell antibodies. Whereas antibodies against pan-ECs, such as anti-CD31 and anti-CD34 antibodies, have been used in evaluation of angiogenesis, these pan-EC antibodies can react not only with "newly forming" vessels but also with normal vessels just trapped within tumour tissues [70]. Other probable reasons for the discrepancies might

be operator-induced. Different investigators might identify different "hot-spots" and thus an operator bias might be encountered. The use of computerised automatic vessel quantification [75, 76] has been presented, and by using a computer automated estimation of MVD, one might say that operator-induced error is minimal, and if error still occurs, these errors occur systematically and thereby minimise bias. Other techniques in determining angiogenesis such as measurement of blood flow in vivo by using colour doppler, positron emission tomography and magnetic resonance imaging have emerged [77]. Recent improvements in the ability to measure circulating angiogenic factors in sera and plasma have led to many publications on various cancer forms [78-81], including lung cancer [82-88]. This approach in determining angiogenesis is attractive, as this approach also is operator-independent. Furthermore, the genomic instability and heterogeneity of tumour cells may explain the clinical observation that the outcomes of patients with tumours in the same pathological or clinical stage, and their response to anticancer therapy, vary considerably. This points to the importance of establishing a molecular-biological profile in patients with lung cancer. A brief historical compilation of breakthroughs in the angiogenesis trial is presented in Table V.

Table V. Historical breakthroughs in the angiogenesis field, with special focus on malignant disease

1787 -	Dr. John Hunter, British surgeon, first uses the term 'angiogenesis' (new blood vessel growth) to describe blood vessels growing in reindeer antler [89].
1971 -	Dr. Folkman hypothesises that tumour growth is dependent upon angiogenesis. His theory, published in the New England Journal of Medicine, is initially regarded as heresy by leading physician and scientists [90].
1975 -	The first angiogenesis inhibitor is discovered in cartilage by Brem et al.. [91].
1984 -	The first angiogenic factor (basic fibroblast growth factor, bFGF) is purified by Shing et al. at Harvard Medical School [92].
1989 -	One of the most studied angiogenic factors, vascular endothelial growth factor (VEGF), is discovered by Ferrara et al. [93]. It turns out to be identical to a molecule called Vascular Permeability Factor (VPF) discovered in 1983 by Senger et al. [94].
1992 -	The first clinical trial of an antiangiogenic drug (TNP-470) begins in cancer patients [95].
1997 -	O'Reilly and colleagues demonstrate nearly total regression of malignant tumours following repeated cycles of antiangiogenic therapy using angiostatin and endostatin [96].
1999 -	Massive wave of antiangiogenic drugs in clinical trials.
2003 -	The monoclonal antibody drug Avastin (Bevacizumab) becomes the first antiangiogenic drug shown in large-scale clinical trials inhibiting tumour blood vessel growth, can prolong survival in cancer patients [97].

Angiogenesis Related Molecules

Many angiogenic growth factors and angiogenesis inhibitors have so far been discovered. A summarised compilation from PubMed is demonstrated in Table VI.

Table VI. A brief summary from a PubMed search on angiogenic growth factors and endogenous inhibitors

Known Angiogenic Growth Factors	**ref**
Angiogenin	98
Angiopoietin-1	99
Developmental locus 1 (Del-1)	100
Fibroblast growth factors: acidic (aFGF) and basic (bFGF)	101
Follistatin	102
Granulocyte colony-stimulating factor (G-CSF)	103
Hepatocyte growth factor (HGF) /scatter factor (SF)	104
Interleukin-8 (IL-8)	105
Leptin	106
Midkine	107
Placental growth factor	108
Platelet-derived endothelial cell growth factor (PD-ECGF)	109
Platelet-derived growth factor-BB (PDGF-BB)	110
Pleiotrophin (PTN)	111
Progranulin	112
Proliferin	113
Transforming growth factor-alpha (TGF-alpha)	114
Transforming growth factor-beta (TGF-beta)	115
Tumour necrosis factor-alpha (TNF-alpha)	116
Vascular endothelial growth factor (VEGF/VPF)	93, 94

Table VI. Continued.

Known Angiogenesis Inhibitors	
Angioarrestin	117
Angiostatin (plasminogen fragment)	118
Antiangiogenic antithrombin III	119
Cartilage-derived inhibitor (CDI)	120
Endostatin (collagen XVIII fragment)	96
Fibronectin fragment	121
Heparinases	122
Interferon alpha	123
Interferon inducible protein (IP-10)	124
Interleukin-12	125
Kringle 5 (plasminogen fragment)	126
Metalloproteinase inhibitors (TIMPs)	127, 128
2-Methoxyestradiol	129
Placental ribonuclease inhibitor	130
Platelet factor-4 (PF4)	131
Prolactin 16kD fragment	132
Proliferin-related protein (PRP)	113
Retinoids	133
Thrombospondin-1 (TSP-1)	134
Troponin-I	135
Transforming growth factor-beta (TGF-b)	136
Vasostatin (calreticulin fragment)	137

In the present thesis, we have investigated VEGF and bFGF serologically as well as several other angiogenesis-related molecules, such as HER-2, EGFR/HER-1, COX-2 and p53 immunohistochemically, and by means of a

cDNA based sequencing technique, we further investigated the mutational status of p53. Moreover, MVD has been calculated automatically with a image analysis system from CD34 and CD105 immunohistochemical stainings. All markers will be discussed in detail later.

VEGF

VEGF (VEGF-A) is a angiogenic factor specific for vessel endothelia; other members in the VEGF-family are VEGF-B, VEGF-C, VEGF-D, VEGF-E and PlGF [138]. The gene knockout has proven to be lethal, not only the homozygous knockout, but also the heterozygous knockout [139]. So far at least 7 different mRNA spliceforms (VEGF121, 145, 148, 165, 183, 189, 206) have been discovered, all generated from the same gene, localised on the short arm of chromosome 6, by alternative splicing [138]. VEGF121 and 165 are soluble; the other isoforms are almost exclusively cell- and matrix-associated [140]. Recently, Bates et al. [141] presented the discovery of a alternatively spliced 165 amino acid isoform which they called VEGF165b [141]. This isoform demonstrated endogenous inhibitory properties when conditioned medium containing the isoform significantly and dose dependently inhibited $VEGF_{165}$-mediated proliferation, migration of endothelial cells, and vasodilatation of mesenteric arteries. This finding might indicate that other alternative spliced isoforms might exist and probably function with different activities. All isoforms share common exons 1–5 and the terminal exon 8, corresponding to the minimally functionally VEGF isoform, VEGF121. Inclusions of exons 6 and 7 by alternative splicing generate the other VEGF isoforms [142]. All VEGF isoforms are capable of binding to the receptor tyrosine kinases KDR/Flk-1/VEGFR-2 [143]. or Flt-1/VEGFR-1 [144]. Ligand binding to the receptor causes a dimerisation and an autophosphorylation is initiated. It seems that only

KDR/Flk-1/VEGFR-2 mediates mitogenic, chemotactic and morphological functions in a ligand-dependent fashion [145].

The function of the different VEGF isoforms seems to depend on which exon is within the isoform. The terminal part of exon 7 has recently been demonstrated to be responsible for binding to the newly discovered co-receptor Neuropilin-1 [146]. Exons 6 and 7 regulate binding to heparin or heparan sulphate proteoglycans [140]. Exon 8 seems to be necessary for the stimulation of mitosis [141, 147]. Exons 3 and 4 seem to be responsible for binding to VEGF-R1 and VEGF-R2, respectively [138]. Exon 3 has also been demonstrated to include the dimerisation domain, whereas exon 1 contains the signal sequence and exon 2 is the N-terminus [148]. VEGF expression is increased by hypoxia [149], pro-inflammatory cytokines such as interleukin-1B [150], and there are indications that also other growth factors, such as platelet derived growth factor (PDGF) [151] and basic fibroblast growth factor (bFGF) [152], enhance VEGF expression and VEGF receptor expression. Oncogenes such as p53 [153, 154] and tumour suppressor genes such as Ras [155] have also been correlated to VEGF-gene involvement. Recent publications indicate that VEGF is the angiogenic factor involved in the angiogenic switch/progression [156], whereas earlier publications reported bFGF as the responsible candidate [157]. Regarding lung cancer, significant correlations between VEGF expression and poor prognosis in NSCLC [158-161] have been presented.

bFGF/FGF-2

bFGF is one member out of at least 22 members in the fibroblast growth factor (FGF) family [162]. Together with acidic fibroblast growth factor (aFGF)/FGF-1, bFGF/FGF-2 is also proposed as prototype [163]. The genome has only one copy for bFGF and has been localised to the short arm of chromosome 4 [164] and, due to different transcription sites on the gene, 5

different isoforms with different molecular weight (18, 22, 22.5, 24 and 34 kDa) have been discovered [165, 166], fig. 3.

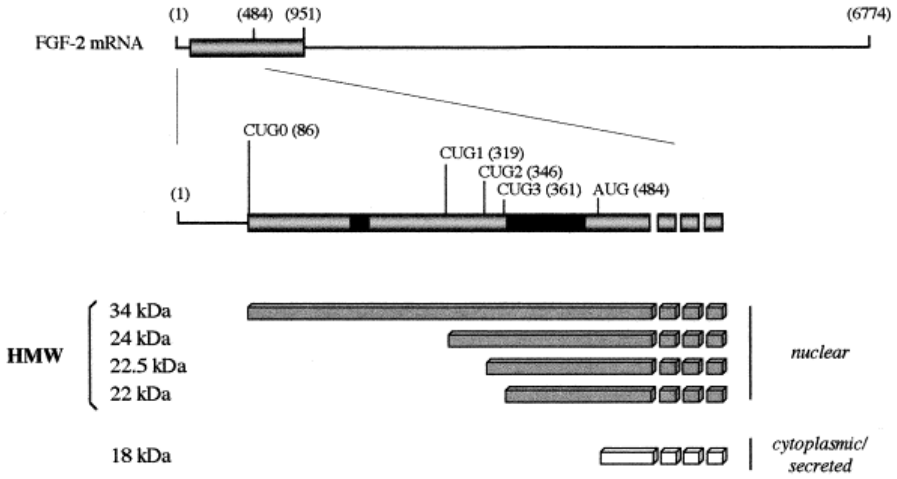


Fig 3. *Due to alternative transcription sites 5 different isoforms of bFGF are generated. Adapted from Delrieu I* [167]

bFGF actions are mediated through three different pathways, partly depending on the molecular weight. The low molecular form, 18 kDa, can activate genes either through the bFGF tyrosine kinase signalling pathways or through internalisation and co-localisation of the activated ligand-receptor complex into the cell nucleus [167]. The high molecular forms probably have a intracrine mode of action as they have nuclear localisation [168], and thereby have the potential of interactions with intracellular molecules before targeting and regulating the expression of specific genes [167].

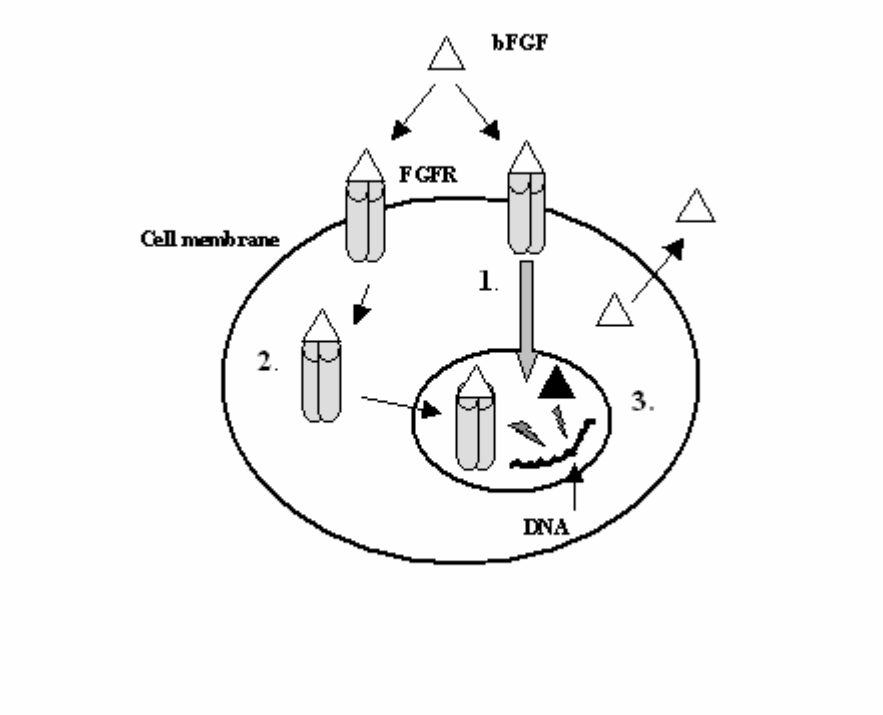


Fig 4. *bFGF signalling pathways. White triangle =18 kDa isoform. Black triangle = high molecular isoforms. 1) Tyrosine kinase signalling. 2) Internalisation. 3) Intracrine mode of action of high molecular forms. Adapted from Delrieu I* [167]

bFGF stimulates not only angiogenesis, it also involved in cell differentiation and proliferation in broad spectrum of mesodermal and neuro-ectodermal cell types and the different isoforms of bFGF display specific patterns of expression depending on tissue, developmental stage or cellular stress conditions [169]. bFGF stimulates wound healing, tissue repair, and haematopoiesis, which includes granulopoiesis, megakaryocytopoiesis, and stem cell survival [170]. bFGF promotes the proper function of the nervous system, which includes postmitotic neurons derived from the cerebral cortex, hippocampus, cerebellum, retina, ciliary ganglion, and spinal cord [171]. The

bFGF has demonstrated angiogenic effects both in vitro [172] and in vivo [173] and appears also to be synergistic when VEGF is expressed concomitantly.
In lung cancer, immunohistochemical expression studies regarding bFGF are sparse and presents conflicting results. In the study from Takanami et al. [174], the authors showed that high bFGF expression was an independent prognostic factor, but FGFR-1 was not. This was contradictory to the data presented by Volm et al. [175], where the authors demonstrated that overexpression of FGFR-1 was significantly linked with poorer survival, but bFGF was not. However, FGFR-1 expression was not a independent prognostic factor.

CD34

CD34 is a cell surface protein, a sialomycin, expressed on haematological progenitor cells and small vessel endothelial cells [176]. The CD34 gene is located on chromosome 1 [177, 178] and is reported to span 26 kb and have 8 exons [179]. The CD34 gene is encoding for a sialomycin of yet unknown function [180]. However, CD34 has been reported to be a phosphoprotein activated by PKC [181] and has also been demonstrated to function as a ligand for L-selectin, resulting in leukocyte attachment at sites of inflammation [182, 183]. Furthermore, CD34 has been suggested to have a role in vessel sprouting, one of the initial steps in angiogenesis, since immunohistochemical studies have revealed increased CD34 expression at tips of vascular sprouts [184]. CD34 has been reported not to be induced by known endothelial growth factors [176], and instead Hellwig et al. [185] report that bFGF and VEGF downregulate CD34 expression, at least in renal cell carcinoma. Recently, Ashara et al [44] managed to isolate endothelial cell progenitors or angioblasts from human peripheral blood using the cell surface antigen expression of CD34 or Flk-1.

In lung cancer, a recent meta-analysis by Mert et al. [66] investigating different endothelial markers such as factor VIII, CD31 and CD34, showed a possible superiority of CD34 in sensitivity with regards to endothelial cell stainings.

CD105

Endoglin, also called CD105, is a homodimeric membrane glycoprotein primarily associated with human vascular endothelium. CD105 is also found on bone marrow proerythroblasts, activated monocytes, and lymphoblasts in childhood leukemia [186]. In 1993, Fernandez-Ruiz et al. [186] showed that the gene, located in chromosome 9, encodes 2 variants of endoglin. The difference is at the extracellular region. Either can a long (47-amino acid cytoplasmic tail) or a short (14-amino acid cytoplasmic tail) tail can be expressed. Both isoforms bind transforming growth factor-beta-1 [187]. Endoglin is a component of the transforming growth factor beta receptor complex, as it binds TGFB1 and TGFB3 with high affinity [188]. The cytoplasmic tail of CD105 has been shown to modulate TGF-ß signalling due to its different interactions with the signalling receptors TßRI and TßRII [189]. CD105 mutations have been linked with a vascular disorder called hereditary hemorrhagic telangiectasia, or Rendu–Osler–Weber syndrome [190]. This syndrome is a family autosomal dominant vascular disorder, which is characterised by the vascular arteriovenous malformations (AVM) that vary in size from 1 mm to several centimetres, located frequently in nasal, gastrointestinal and cerebral vascular beds.

Expression studies using CD105 as the endothelial marker in lung cancer have demonstrated the superiority of CD105 over CD34 [70, 191].

EGFR(HER-1/erbB-1) and HER-2/neu (erbB-2)

The ErbB gene family consists of four members, of which EGFR and HER-2 are two. All members share sequence homologies and function as transmembrane glycoprotein tyrosine kinases [192]. EGFR gene is located on chromosome 7 [193] and HER-2 gene is located on chromosome 17 and has extensive sequence homology to EGFR [194]. The ligands for EGFR are epidermal growth factor (EGF) and transforming growth factors (TGFs) [195, 196], whereas the ligand for HER-2 still remains unclear [192]. All receptor members of the erbB gene family can form dimers with each other upon ligand-binding, however it seems that the intensity in the transferred signal differs depends on which receptor complex that is assembled. HER-3 has no tyrosine kinase activity, so homodimerisation between these isoforms results in no signal, whereas homodimerisation between HER-1 receptors and between Her-4 receptors results in a weak signal. However, when there is heterodimerisation between HER-2 and either of the other receptors, a strong signal is transferred [192]. The cell effect upon receptor activation ranges from differentiation, cell division and migration, adhesion, transformation and apoptosis. Effect depends upon which cells are affected, on mesoderm or ectoderm origin, and which erbB dimer that is activated. Homodimers are less mitogenic and transforming, compared with heterodimers and, especially HER-2 containing heterodimers [192]. Regarding lung cancer, HER-2 overexpression can be detected in patients with NSCLC, which in turn, correlates with a poor outcome [197-199]. On the other hand, EGFR overexpression in NSCLC has been reported to be around 50% [199, 200], but EGFR overexpression has rarely been correlated to clinical or prognostic factors regarding NSCLC [201].

COX-2

Cyclooxygenase (prostaglandin-endoperoxid synthase) is an enzyme involved in the production of prostaglandins and eicosanoids from arachidonic acid [202]. Two isoforms have been identified, one housekeeping gene located on chromosome 9 where a 2.8-kb mRNA [203] encodes isoform Cox-1, and a mitogen-inducible form on chromosome 1 where a 4.5-kb mRNA [204] encodes isoform Cox-2. Cox-1 is believed to be responsible for Thromboxane A2 inhibition in platelets and the unwanted side-effects, such as the interruption of the gastric mucosa protection, whereas Cox-2 is believed to be responsible for the desired drug effect as anti-inflammatory properties [202]. Cox-2 is linked with the characteristics featuring carcinogenesis. Tsujii et al. [205] demonstrated with a migration and a differentiation assay that Cox-2 expressing colon cancer cell lines co-cultured with human umbilical vein endothelial cells (HUVEC) expressed prostaglandins, pro-angiogenic factors and stimulated endothelial cell migrations and tube formation. When a selective Cox-2 inhibitor (NS-398/Celecoxib) was added, tube formation and angiogenic factor expression were inhibited. In lung cancer, Cox-2 is frequently overexpressed and this overexpression has been demonstrated as a marker of poor prognosis in resected stage I NSCLC [206].

p53

The p53 gene has been localised to the short arm of chromosome 17 and has 11 coding exons [207]. The corresponding protein of the p53 gene is a 53-kDa nuclear phosphoprotein consisting of 393 amino acids [208]. Under normal circumstances, the p53 protein is maintained at low levels within the cells due to the rapid degradation by ubiquitin-dependent proteolysis. The ubiquitin-dependent proteolysis is activated by a direct transcriptional target

MDM2 [209]. MDM2 can also bind to the activation domain of p53 and thereby inhibits the p53 to stimulated transcription [210]. Nevertheless, the stability and degradation of p53 is not entirely dependent on the MDM2-pathway. Other proteins, such as JNK (jun-N-terminal kinase) [211] and papillomaviruses, E1B and E6, are also capable of inducing p53 degradation [212]. If the cell is exposed to stress, such as DNA damage [213], oncogene activation [214], hypoxia [215] as well as cytokine activation, viral infections, heat shock or metabolic changes [216], a rapid elevation of the p53 protein is elicited. The p53 protein results either in cell cycle arrest, allowing DNA repair [217], or cell death through apoptosis. p53 is linked to caspase activation through several transcriptional targets such as Bcl-2, a gene that can suppress apoptosis in a range of cell types [218], and Bax, a gene that promotes apoptosis [219]. When p53 induces Bax and inhibits Bcl-2, the effect is a mitochondrial release of Cytochrome C which in turn activate APAF-1 and apoptosis is initiated. There are numerous studies investigating p53´s prognostic impact in NSCLC. In a recent publication from Mitsudomi et al. [220], an effort was made to compile data into a meta-analysis is performed. The authors showed a statistically poorer prognosis in patients with adenocarcinomas if p53 protein overexpression is demonstrated. For squamous cell carcinomas, the trend is towards the same assumption, but is not statistically significant.

Aims of the investigations

I. This study was performed to investigate the prognostic impact of circulating VEGF and bFGF in preoperatively collected sera from patients with operable NSCLC.

II. Based on the findings in study **I**, this study was aimed to investigate if serum VEGF or bFGF have prognostic information in patients with inoperable NSCLC and might be used as tumour markers and thus be suitable for therapy monitoring.

III. This study investigated the interrelationships and prognostic relevance of potentially new clinical targets, HER-2, EGFR, COX-2 and their correlation to microvessel density and survival in operable NSCLC. Immunohistochemical techniques were used and the estimation of MVD, determined from CD105 stainings, was done fully automatically. The study comprised of tumour samples from patients with resected NSCLC.

IV. In this study, we investigated tumour specimens from patients with resected NSCLC regarding the immunohistochemical expression of p53, CD34 and CD105, and further sequenced the p53 gene by cDNA sequencing in these tumours. These immunohistochemical and sequencing data were then correlated with the data sampled earlier regarding serum VEGF and bFGF levels (**I**), with the aim to predict recurrences in NSCLC, alone or together in a molecular-biological profile.

Material

Patients

In **Paper I**, 58 patients with a verified diagnosis of resectable NSCLC, stage I and IIIa, were included. In **Paper II**, 73 patients with a verified inoperable NSCLC, stage IIIb and IV, were included. In **Papers III and IV,** 53 paraffin imbedded tumour samples from a population of patients that were resected for NSCLC during 1993-1996 were selected for immunohistochemical studies. Furthermore, in **Paper IV**, 32 fresh frozen tumour samples from the operation were analysed for their p53 mutational status by cDNA sequencing. In all studies, clinical parameters were scored by reading patient charts.

Serum samples

In **Paper I**, serum samples collected during 1993-1996 from 58 patients with a verified diagnosis of NSCLC were included. All patients had to be planned for curative intended surgery and made an informed consent regarding storage of tumour and serum samples before surgery. Blood samples were collected in 7 ml serum tubes without additive and stored at -20 ^{o}C until analysed. In **Paper II**, a total of 460 serum samples from 73 NSCLC patients were collected at the Department of Oncology, Uppsala Akademiska Hospital, during 1983 to 1992. Prior to sampling, all patients gave informed consent and the study has been reviewed and approved by the research ethics committee, no: 02-010, Uppsala University, Uppsala, Sweden. Serum samples have been stored at –70 ^{o}C until analysed and all sampling have followed the standard routines. In **Paper IV**, data from 53 of the serum samples collected in **Paper I** were correlated with p53 immunohistochemical and sequencing data and MVD.

Methods

Tumour volume calculations

Primary tumour volume estimations (**Papers I & IV**), only including measurable disease involving the lungs, were re-evaluated from CT-scans in 62% of the cases. In the other cases, new measurements of tumour volume were made from pulmonary X-rays. All calculations regarding tumour volume were made using the formula: 4 π *X-radius*Y-radius*Z-radius/3.

VEGF and bFGF calculations

For detection of circulating VEGF and bFGF in sera (**Papers I, II & IV**), immunosorbent assays from R&D Systems were used (QuantikineTM human VEGF and QuantikineTM HS human FGF basic, R&D Systems, Minneapolis, MN, USA). The principle of these assays employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF/bFGF is pre-coated onto a microplate. Standards and samples are pipetted into the wells and any VEGF/bFGF present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for VEGF/bFGF is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of VEGF/bFGF bound in the initial step. The colour development is stopped and the intensity of the colour is measured. All preparations and calculations followed the manufacturer's recommendations, and were performed at the Department of Clinical Chemistry.

VEGF preparations **Basic FGF preparation**

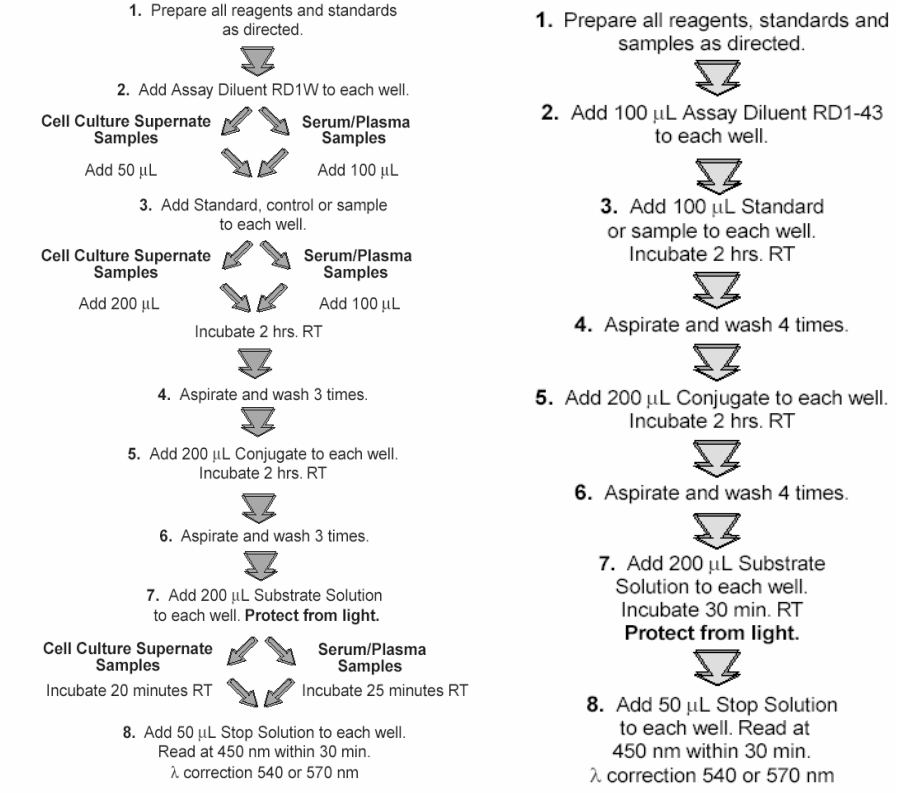


Fig 5. The assay procedure summaries adapted from R&D system brochures.

An elevation of VEGF and bFGF levels was defined as being greater than the 95th percentile value in a normal control subject group provided by the manufacturer. This resulted in a cut-off value for VEGF at 500 ng/L (mean 224 ng/L range 62-707 ng/L). bFGF calculations rendered a cut-off value for bFGF at 7.25 ng/L (mean 2.9 ng/L range 0.3-31.2 ng/L).

Immunohistochemistry (IHC)

In **Paper III**, The tumour samples were sectioned into 4-µm paraffin sections and placed onto Superfrost/plus® slides (Mentzel, Germany), deparaffinized in xylen and rehydrated in graded alcohols. For each antibody, details such as dilutions and choice of antigen retrieval are specified in Table VII. Both a manually operated and a fully automated immunohistochemistry were used. In the manual immunohistochemistry, two antigen retrieval methods were used: slides were either immersed in 0.01 M citrate buffer, pH 6.0 or in 0.002 M EDTA containing 0.05 M Tris, pH 9.0, and boiled for 7 minutes in a Decloacing chamber® (Biocare Medical, Walnut Creek, CA, USA). Endogenous peroxidase was blocked in 0.3% H_2O_2 in PBS for 20 minutes. To reduce non-specific binding of the primary antibody, sections were pre-incubated in 0.5% BSA-c® (Aurion, Wageningen, Netherlands) in PBS. Sections were incubated with the primary antibody for 16 h at 4°C, followed by incubation for 45 minutes at 20°C in EnVision® (DAKO). Alternatively, a biotinylated horse anti-goat link-antibody (Vector, Burlingame, CA, USA) was used, followed by peroxidase-labelled streptavidin biotin-complex (DAKO). Diaminobenzidine (Sigma, St. Louis, MO, USA) or glucose oxidase (Sigma) were used as chromogen and Harris hematoxylin (Sigma) or lightgreen for counterstaining. Samples with known expression of HER-2 (breast carcinoma and a commercially available (DAKO) control slide, containing cultured cells (SK-BR-3, MDA 175 and MDA231) and EGFR (placenta) were used as positive controls. For negative controls we used parallel sections of tumour samples with the primary antibody omitted and replaced with PBS. The automated immunohistochemistry was done in a fully automated immunostaining instrument, Ventana Benchmark® (Ventana Medical Systems Inc., Tucson,

AZ, USA). Deparaffinization, dehydration, and antigen retrieval (enzymatic or heat induced) were done in the instrument, using prediluted solutions from the manufacturer. A standard diaminobenzidine (DAB) detection kit (Ventana) was used according to instructions from the manufacturer. The slides were manually counterstained in Harris haematoxylin (Sigma, St. Louis, MO, USA). Finally, the slides were dehydrated through graded alcohols to xylen and mounted in organic mounting medium (Pertex, Histolab, Gothenburg, Sweden).

Table VII. Antibody characteristics and immunostaining procedures

Antibody	Clone	Source	Staining & dilution		Epitope retrieval	Counter-staining
			Manual	Ventana		
CD105	4G11	Novocastra Newcastle upon Tyne, UK	1:25		HIER in Tris-EDTA buffer, pH 9.0	Lightgreen
COX-2	Poly-clonal	Santa Cruz Biotech., Santa Cruz, USA	1:1000		HIER in 10mM citrate buffer, pH 6.0	Harris hema-toxylin
EGFR	31G7	Zymed labs., South San Francisco, CA, USA		1:20	Enzymatic	Harris hema-toxylin
HER-2	Poly-clonal	DAKO, Glostrup, Denmark		1:300	HIER in prediluted CC1 buffer	Harris hema-toxylin

In **Paper IV**, the immunohistochemistry was also done either manually or in a fully automated immunostaining instrument, Ventana Benchmark® (Ventana Medical Systems Inc., Tucson, AZ, USA). Deparaffinisation, dehydration, and antigen retrieval (enzymatic or heat-induced) was done in

the instrument. A standard diaminobenzidine (DAB) detection kit (Ventana), was used according to the instructions from the manufacturer. Paraffin sections of 4-µm thickness were placed onto Superfrost/plus® slides (Mentzel, Germany), deparaffinised and rehydrated in graded alcohols. Two antigen retrieval methods were used: slides were either immersed in 0.01 M citrate buffer, pH 6.0 and boiled in a microwave oven, 750 W, for 2x5 minutes or immersed in 0.002 M EDTA containing 0.05 M Tris, pH 9.0, and boiled for 7 minutes at full pressure in a Decloacing chamber® (Biocare Medical, Walnut Creek, CA, USA).

For the endothelial markers, CD34 (Serotec) and CD105 (clone 4G11; 1:25; Novocastra, Newcastle upon Tyne, UK), the manual immunostaining protocol was used. Briefly, endogenous peroxidase was blocked in 0.3% H2O2 in PBS for 20 minutes. To reduce non-specific binding of the primary antibody, sections were pre-incubated in 0.5% BSA-c® (Aurion, Wageningen, Netherlands) in PBS. The primary antibody was incubated over night at 4°C followed by incubation with a goat anti-mouse peroxidase-conjugated dextran polymer, Envision® (DAKO, Glostrup, Denmark) at room temperature for 30 minutes. The slides were developed in glucose oxidase (Sigma, St. Louis, MO, USA) and counterstained in lightgreen.

The mouse antihuman p53 (clone DO-7; 1:200; Dako, Glostrup, Denmark) antibody, was immunostained in the fully automised Ventana Benchmark® (Ventana Medical Systems Inc) immunostaining instrument. The slides were manually counterstained in Harris hematoxylin (Sigma). Finally, all slides were dehydrated through graded alcohols to xylen and mounted in organic mounting medium (Pertex, Histolab, Gothenburg, Sweden).

Evaluation of the HER-2-, EGFR- and COX-2-immunostaining was performed by two of the authors in **Paper III** (D.B & H.N). Samples were reviewed together in a multi-headed microscope and a consensus was scored. HER-2 and EGFR expression was scored semi-quantitatively as follows:

1) More than 67% of tumour cells should be stained. 2) The staining intensity should be moderate to intense (2+ or 3+). 3) The staining pattern should be membranous, with or without concomitant cytoplasmic staining [221]. In addition, in a few cases a strong HER-2 basolateral staining was present and scored as 2+. This scoring technique has earlier been described by Hirsch et al [222]. Regarding COX-2 expression, smooth muscle staining, which was present in all samples, served as internal control and a positive staining was scored if staining was more intense than the internal control and present in more than 50% of the tumour cells [223].

Immunoreactivity was classified semi-quantitatively in **Paper IV**. Several investigators (L.S, M.B and D.B) reviewed and scored slides simultaneously in a multi-headed microscope by estimating the percentage (0, 1-10, <50 and >50%) and staining intensity of tumour cells (0-3+) showing characteristic staining. A consensus between the investigators was obtained at the time of the reading. The cut-off value for tumour cell staining used in this study was defined as high p53 overexpression if >10% of the tumour nuclei were stained. For determination of the cut-off value, we used our previously published cut-off regarding lung cancer [224]. All the immunohistochemical preparations were done at the Department of Genetics and Pathology.

Microvessel quantification

The invasive front was outlined by an experienced pathologist (H.N) on hematoxylin eosin stainings. A minimum of 10 consecutive images were captured. The 756x572 pixel colour images with 3x256 grey levels were captured by a Sony DXC-151 colour video camera attached to a standard Olympus BH-10 microscope, using a x20 objective. This gave a final magnification of x50 and a pixel size of about 0.8 μm for a wavelength of 550nm. For all images, Köhler illumination was maintained and the aperture

iris diaphragm ring was fixed to 0.5. Thereafter, automatic quantification was performed without influence from the operator. The area measured on each image corresponded to 0.22 mm^2. In **Paper III**, a minimum of 8 legible images was set as a cut-off for calculations of MVD according to CD105 stainings. This cut-off rendered 43 evaluable tumour samples for automatic calculations. The cut-off was set according to previous publications with the same software on other carcinomas [75]. In **Paper IV**, the cut-off was lowered as a minimum of 3 legible images was set as cut-off for MVD automatic calculations for CD34 and CD105. With this cut-off, 51 out of 53 tumour samples were evaluable for automatic MVD quantifications. After image capturing, a mean vessel number per mm^2 was calculated (mMVD) (**Papers III & IV**). A mean CD34/CD105 fraction per tumour was also estimated (**Paper IV**). The automatic quantification software was developed at the Centre for Image Analysis, Uppsala University, Sweden and is presented in detail elsewhere [225].

Sequenced-based analysis of p53

In **Paper IV**, cDNA based sequencing of the entire p53 gene was performed on 32 fresh frozen tumour specimens. The technique has previously been described in detail [226]. All tumour samples were cryostat sectioned and stained by hematoxylin to allow an individual reference diagnosis of each section. All the stained slides, including formalin-fixed and paraffin embedded sections from the same cancer were reviewed and selected by a senior pathologist (L.S) at the Department of Pathology, Uppsala University. From each frozen tumour sample, four consecutive 10-µm thick sections were cut and placed on standard objective slides to facilitate a smooth transfer of the microdissected cells. All sections were hematoxylin stained to ensure correct selection of target cells. Three sections were used for

microdissection and one served as a control for confirming the accuracy of the microdissection and the precise morphological identification. Sections were immediately fixed with 95% ethanol for 10 min, followed by Mayer's Hematoxylin staining for 20 seconds prior to microdissection. RT-PCR was performed by using Ready To Go RT-PCR Beads Kit (Amersham Pharmacia Biotech Inc. NJ. USA). Immediately prior to adding the RT-PCR mix, the sample tube was placed on a magnet and the supernatant was discarded. Thereafter the beads were resuspended with 50 µl of room temperature DEPC-treated water and transferred to a 0.2 ml tube with Ready to go RT-PCR beads. The sample was incubated on ice until the beads dissolved, and then transferred to a thermal cycler and incubated at 50°C for 5 min, 42°C for 30 min and 95°C for 5 min to inactivate the reverse transcriptase and completely denature the template. Then the cDNA library was ready for RT-PCR. All the p53 gene specific primers were obtained from the DNA Technology (Aarhus, Denmark). Four pairs of primers were used for the entire p53 gene amplification. The sequences of these primers are Fragment 1: 5'-GAC ACG CTT CCC TGG ATT GGC, 5'-GCA AAA CAT CTT GTT GAG GGC A; Fragment 2: 5'-GTT TCC GTC TGG GCT TCT TGC A, 5'-GGT ACA GTC AGA GCC AAC CTC; Fragment 3: 5'-TGG CCC CTC CTC AGC ATC TTA, 5'-CAA GGC CTC ATT CAG CTC TC; Fragment 4: 5'-CGG CGC ACA GAG GAA GAG AAT C, 5'-CGC ACA CCT ATT GCA AGC AAG GG. The lengths of PCR amplified fragments were 453 bp for Fragment 1, 370 bp for Fragment 2, 482 bp for Fragment 3 and 345 bp for Fragment 4 [226]. Thermocycling was modified from the same methodology as reported. Briefly, PCR reactions were carried out in a thermocycler (Applied Biosystem, Norwalk, Connecticut) using a mixture (20 µl) containing the cDNA obtained from RT reaction, 10 mM Tris-HCl (pH 8.3), 2.0 mM MgCl2, 50 mM KCl, 0.2 mM dNTP; 10 pM of each primer and 1 units of Platinum polymerase (Life technology, USA).

Temperature cycles and times for PCR reactions were: denaturation at 94°C for 20 sec, annealing at 58°C for 40 sec, and extension at 72°C for 45 sec. Each PCR reaction was preceded by a 2 min denaturation at 94°C, and the final cycle was followed by a 10 min extension at 72°C. Altogether there were 40 cycles for PCR amplification. All the PCR products were purified before the sequencing reaction. Before sequencing, an enzymatic purification method was used. Six µl of PCR products was mixed with 1 µl of Exonuclease I (10 unit/µl) and 1 µl of Shrimp alkaline phosphatase (1 unit/µl) (Amersham-Pharmacia Biotech, Uppsala, Sweden). The mixture was placed on a PE 9600 thermocycler at 37°C for 20 min to digest the single strand nucleotide, and then at 80°C for 15 min to inactivate the enzyme. ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) was used for the sequencing of PCR products. Automatic sequencing was performed with the ABI 377 (PE Applied Biosystems, Foster City, CA). Sequencing was analysed from both strands in order to validate laboratory findings. When a mutation was observed in one strand, we confirmed its presence by sequencing the other strand In addition, if there was any ambiguity we confirmed the mutations identified by re-analysing the original DNA template. To define a mutation, we analysed each nucleotide peak in Sequencer (Gene Code, USA) and used a rise of 15% signal above the background threshold level as a cut-off for the presence of mutation. The sequenced based analysis was done at the Department of Genetics and Pathology.

Statistics

In **Paper I**, Survival was estimated using the Kaplan-Meier product limit method as the univariate analysis, and Cox regression as the multivariate analysis, in order to investigate if certain factors had a significant effect on survival. Pearson's product movement, Spearman rank order correlations and Mann-Whitney U-test were utilised for tests of associations between factors. A 5% significance level was used.

In **Paper II**, an estimation method was needed which takes into account the changes in the blood samples during a survival period. A model used in survival analysis is the proportional hazard regression model introduced by Cox in 1972 [227]. This model can be extended to take into account changes in variables during the remaining lifetime. The model used in this study is based on the levels of bFGF, VEGF and platelet counts in sera for each blood sample. The levels are assumed to be constant between two different blood samples or to time of death. In the analysis, a cut-off for platelet counts of 400 x 10^9 is used, where samples with values below the cut-off are regarded as normal values. The levels of angiogenic factors are analysed separately for samples with normal and elevated platelet counts. The survival time was defined from diagnosis to death. Death due to other causes than lung cancer was censored. Differences in VEGF and bFGF levels among prognostic factors were tested by using a one-way analysis of variance (ANOVA). Throughout this study a statistical significant rejection level of 5 percent is used.

In **Paper III,** Fisher´s exact two-tailed test was used to test associations in categorical variables. The survival functions were estimated with the Kaplan-Meier product-limit method and the median survival time was estimated with linear interpolation in the survival function. Cox-regression analysis was applied to study if certain factors had an impact on survival. In

cases where there was only one dichotomous explanatory factor, the results of the Cox-analysis are the same as from the log rank test. Throughout this study, a 5 % significance level is used in the statistical tests.

In **Paper IV,** the Fischer exact two-tailed test and the Chi-square with Yates correction and Spearman rank order correlations were applied, when appropriate, to examine the association of variables. Differences in continuous variables between dichotomous variables were tested using a one-way analysis of variance (ANOVA). Prognostic variables were then included in a regression analysis to study if certain factors had an impact on the recurrence rate. Throughout this study, a 5 % significance level was used in the statistical tests.

Results

Paper I

As growing evidence was accumulating regarding the additive prognostic information MVD had in lung cancer, the search for a circulating angiogenic factor with close correlation with MVD began. In 1998 [228] our group published one of the first articles regarding the prognostic value of circulating VEGF and bFGF in sera from NSCLC patients. The study was not conclusive, probably due to heterogeneity regarding stage in the studied population. However, interesting trends could be demonstrated, indicating a value of continuing to analyse VEGF and bFGF in sera from NSCLC patients. So, in **Paper I**, we investigated circulating VEGF and bFGF in sera in a population with clinically limited potentially resectable NSCLC. Fifty-eight patients were included. Although this material also involved stage IV (n=3) patients, significant correlations between VEGF and tumour volume and overall survival were demonstrated. Sixty-seven percent (9/12) of the patients with elevated levels of VEGF relapsed, compared with 46% (21/46) who relapsed with normal VEGF levels, and the difference proved not to be significant in statistical analysis (P=0.07). bFGF, on the other hand, correlated significantly with recurrent disease. The recurrence rate for patients with elevated bFGF levels was 78% (14/18), compared with patients with normal bFGF levels who had a recurrence rate of 40% (16/40). Moreover, bFGF correlated also with survival when used as a continuous variable. In addition, bFGF proved to be the only independent significant factor in this population of patients. The number of patients with both angiogenic factors elevated simultaneously was too small to enable any conclusions to be drawn. Both angiogenic factors correlated to each other and with platelet counts.

Paper II

In this study, we investigated the potential that serum VEGF and bFGF might have as tumour markers in inoperable NSCLC.

Seventy-three patients were included in this study, stage IIIa and stage IIIb were the most common stages (79 %). A significant difference could be demonstrated regarding bFGF levels between stage IIIa and IIIb. Stage IIIa mean bFGF levels (n=267) with corresponding 95 % confidence interval (CI-95) was 8.9 ng/L (CI-95 7.6-9.9). In stage IIIb patients the corresponding bFGF level (n=138) was 11.5 ng/L (CI-95 9.4-13.5), p=0.015. Disappointingly, no prognostic information could be demonstrated in the first statistical analysis on the whole population regarding VEGF and bFGF. Due to the bias platelets might have upon VEGF levels [229, 230], as discussed earlier, subgroup analysis was performed. The number of patients in the statistical analysis, however, was reduced on account of all patients not having co-sampled values of VEGF/ bFGF and platelet counts. Pre-treatment bFGF levels in patients with normal platelet counts were correlated to poorer survival, p-value=0.047. During chemotherapy, each rise of one unit bFGF corresponded to a hazard ratio of 4.06 (p=0.022). Elevated levels of VEGF after radiotherapy significantly correlated to good prognosis (p=0.023), whereas VEGF levels during radiotherapy demonstrated a trend in the same direction, but did not reach statistical significance (p=0.086).

Paper III

In this study, we aimed to investigate the prognostic information of three putative markers for targeted therapies in resected NSCLC: HER-2, EGFR and COX-2. Furthermore, we also investigated the interrelationships of these markers with angiogenesis, estimated with MVD calculated automatically from CD105/Endoglin immunostainings. Fifty-three paraffin embedded

tumour samples were included in this study. HER-2, EGFR and COX-2 overexpressions were demonstrated in 15%, 30% and 40% of the tumours. None of the 22 squamous cell carcinomas were scored as having a positive HER-2 staining. Three squamous cell carcinomas had 1+ stainings, whereas the rest (n=19) had no staining. Overexpression of HER-2 demonstrated a trend towards a poorer survival, p=0.10. When HER-2 stainings were stratified between histological sub-types, the difference almost reached statistical significance, p=0.07.

As concerns EGFR, ten out of 22 (45%) squamous epithelial carcinomas were positive for EGFR compared with 4 of 22 (9%) in the adenocarcinomas. However, these differences proved not to be statistically significant.

Regarding COX-2 stainings, only 2 out of 22 (9%) squamous cell carcinomas scored as positive compared with 12 out of 22 (55%) adenocarcinomas. This difference between histological sub-types proved to be statistically significant. COX-2 expression correlated with HER-2 expression and demonstrated a trend when correlated with MVD calculated from CD105 stainings (p=0.10).

Median survival in HER-2+/EGFR- was 634 days compared with 1570 days in other HER-2/EGFR combinations. Among 5-year survivors (n=23), there were 17 HER-2- /EGFR- cases compared with only one HER-2+/EGFR- case. Due to the pre-analytical cut-offs, described in detail in the Methods section, for determining readability, only 41 tumour samples were available for statistical analysis on the CD105 stainings. Mean MVD with corresponding 95 % confidence interval (CI) in CD105 was 217 vessels per mm^2 (CI: 187-248). From the result of this study, we concluded that differential expression patterns in HER-2, EGFR and Cox-2 expression exist between different types of histology and that treatment with HER-2 antagonists, might be a future treatment of NSCLC, especially in patients

with primary tumours overexpressing HER-2 without a concomitant overexpression of EGFR.

Paper IV

In this study, we further investigated the same material as in **Paper III**, but now we investigated 2 different endothelial markers as determinants for the angiogenic potential. As previously described, different cut-offs were used, and these data were correlated with the serum fractions of VEGF and bFGF and the immunohistochemical expression of p53. Moreover, we sequenced the whole coding region of the p53 gene in 32 tumour samples for the presence of p53 mutations (exons 2-11) using a cDNA technique.

The primary aim of this study was to investigate if a molecular biological profile with angiogenic factors analysed from the serum fraction, mean MVD determined by immunohistochemistry and p53, investigated either immunohistologically or by sequencing techniques, could predict recurrences. There were 36 (68%) males and 17 (32%) females included in this study. A mean MVD could be calculated in 51/53 tumour samples. The endothelial markers significantly correlated to each other, $p<0.001$. Levels of either VEGF or bFGF were not significantly correlated to mean MVD of either CD34 or CD105. Mean MVD of either CD34 or CD105 were not significantly different depending on recurrent disease or not ($p=0.41$ and 0.78, respectively). However, the survival difference was substantial, numerically, but not significantly, in the CD105 group with a cut-off at the upper quartile number per vessel (>261) mm^2.

Immunohistochemical overexpression of p53 was demonstrated in 49%, among the 32 tumours that were sequenced for p53 mutations, 12 samples harboured a mutation. p53 expressions demonstrated a positive significant correlation with mean MVD estimated from CD105 stainings, $p=0.043$. Eighteen out of 27 (67%) patients with p53 negativity, experienced a relapse

compared with patients with p53 positivity, who experienced relapse in 37% (9/26). This difference proved to be significant, p=0.029. However, p53 expressions only reached a borderline significant survival difference, p=0.057. When plotting CD105 mean MVD levels with survival time, a graphically potential clustering could be demonstrated.

General Discussion and Future Perspectives

Lung cancer is the most common form of cancer worldwide [1]. The outcome of treatment for primary lung cancer treatment is still poor, but science pushes the 5-year survival rates higher and higher. The best way of fighting lung cancer is by primary prevention with information on the risks with tobacco consumption and the beneficial effect of terminating smoking. This is hard work and is carried out in Sweden with great dedication, but the impact on Swedish youth is not solid.

This thesis focuses on cancer patients with the aim of finding new prognostic factors and molecular-biological profiles that are more suitable for a more aggressive therapy than those applied by current treatment schedules.

In early stage lung carcinomas, where the highest rates of cure might be anticipated, thoracic surgery is the offered treatment. Patients not suited for surgery, either due to medical illness or not wanting surgery, are offered different combinations of radio-chemotherapy regimes, as discussed in the introduction.

In locally advanced (IIIa and IIIb) primary lung cancer, chemotherapy has an indisputable place. Chemotherapy is preferably used in different combinations with radiotherapy (sequential, concomitant) [12, 19, 29]. In Sweden, the recommended treatment is the sequential use of 2-3 cycles of platinum based chemotherapy before radiotherapy [7]. The use of radiotherapy with concomitant platinum compounds, or other newer agents such as gemcitabine, vinorelbine and taxanes as radiosensitizers is recommended to be used in clinical controlled trails [7]. Regarding advanced disease, convincing evidence supporting the role of chemotherapy [12, 20-28] has been published, but a standard regime regarding which of the newer drugs that

should be combined with platinum compounds cannot, yet, be recommended.

As the field of angiogenesis research was undergoing explosive growth in the late 90´s, and the development of ELISA tests made it possible to analyse circulating angiogenic factors in clinical blood samples, we set out to investigate the prognostic potential of circulating angiogenic cytokines, VEGF and bFGF in two populations of lung cancer patients (operable and non-operable). We demonstrated that serum fractions of VEGF and bFGF can be used as prognostic factors in locally advanced and advanced lung carcinomas, **Paper II**. Pre-treatment bFGF levels in patients with normal platelet counts were correlated to poorer survival, and during chemotherapy each rise of one unit bFGF corresponded to a poorer survival with a hazard ratio/unit bFGF of 4.06 (p=0.022). Contradictory to the negative impact of high bFGF levels on survival, high VEGF levels in patients with normal platelet counts after radiotherapy were correlated to good prognosis. This finding was intriguing, as we hypothesized that patients that responded to treatment with an elevation of either VEGF or bFGF would have a poorer prognosis compared with patients that retained, or lowered, their levels. A hypothetical explanation might be that VEGF elevation indicates that a cell survival response is initiated, regarding both tumour cells and endothelial cells [231, 232], which indirectly might indicate a tumour responsive to treatment. The significant correlation was not demonstrated in samples collected during radiotherapy. However, a trend could be demonstrated in this setting and thus might be consistent with the hypothesis that a VEGF elevation after radiotherapy was a stress-reaction. In a study by Yoshiji et al [233], VEGF was demonstrated to be of critical importance only in very small tumours, and when tumours reached a certain volume other growth factors could sustain tumour growth. This finding might indicate that tumours in our study might be more bFGF "sensitive", as the study population comprised of

patients with relatively large tumours and, in conjunction with the observations by Yoshi et al. [233], might explain the discrepancies shown in the statistical analysis regarding survival. One further observation in the study worth pointing out is the significant differences between bFGF levels stratified into stage IIIa and stage IIIb patients. Stage IIIb patients had higher levels of bFGF compared with stage IIIa patients. These findings are interesting, as ongoing studies with the aim of a downstaging with chemotherapy before surgery, might have another marker for evaluation of the efficacy, other than the already established techniques.

The prognostic value of analysing serum VEGF and bFGF in operable lung carcinomas was addressed in **Paper I**. Significant correlations between VEGF and tumour volume and overall survival were demonstrated. High VEGF levels reached only a trend in a correlation with recurrences, p=0.07. bFGF, on the other hand, correlated significantly with recurrent disease and survival. In addition, bFGF was also the only independent significant factor in this study. Our data indicate that both angiogenic cytokines are involved in tumour progression, but possibly in different fashions. VEGF might act as the initial angiogenic factor, overexpressed by hypoxia or mutated p53, and bFGF as a second growth factor responsible for continuous transformation capacities and angiogenic stimulation. The present assumption has been investigated by Fang et al. [156] in chondrosarcoma nodules, demonstrating that expression of VEGF and bFGF alters during time. VEGF acts as an angiogenic switch and decreases after neovascularisation where, in striking contrast, bFGF levels rise. The same kinetic profile of EC mitogens have been presented by Yoshiji et al. [233] when they studied s.c. growth of a breast carcinoma cell line. It is worth pointing out that in both studies (**Papers I & II**), there was a close correlation between both angiogenic cytokines and platelets, and before publishing both these studies a discussion regarding the value of measuring VEGF and bFGF in sera had been taking place [229, 230] due

to the possibility of the bias platelets might have on the serum fraction of VEGF, as a result of platelets being implicated to have a role as a "VEGF-donor" [230]. Serum VEGF has been analysed from NSCLC patients in several studies. The diagnostic [87], prognostic [84, 85, 228, 234, 235] and predictive values, with regards to surgery [234, 236] and treatment for advanced lung cancer [86] (Brattström et al., accepted Lung Cancer) have been studied. Data are from relatively small study populations and in two studies, further disturbing the results, mixed histologies (SCLC and NSCLC) were analysed [83, 86]. The value of analysing plasma VEGF in NSCLC is not yet conclusive when addressing its prognostic and predictive values [237-239]. Altogether, data are accumulating in support of the serum fraction possible being the most clinically relevant to study [240-242]. The value of the circulating fraction of bFGF has not been studied to the same extent as VEGF, but this angiogenic cytokine demonstrates even stronger correlations with survival [234, 243]. A compilation of the studies investigating circulating VEGF and bFGF in NSCLC is demonstrated in Table VIII.

Table VIII. A compilation of studies investigating circulating VEGF and bFGF in NSCLC recognized through a PubMed search

Author	Angiogenic factor	No. of patients	Results and reference
Tamura	p-VEGF	160	The diagnostic value of plasma VEGF was better than that of CYFRA and similar to that of CEA [238].
Tamura	p-VEGF	97	P-VEGF correlates with MVD and intratumoural VEGF levels [87].
Kishiro	p-VEGF	28	Untreated patients p-VEGF was higher than 20 control, 35 benign lung disease and 10 lung cancers [239].
Roselli	p-VEGF	65	P-VEGF correlated to proteins involved in platelet and coagulation activation [237].
Imoto	s-VEGF		S-VEGF was higher in T3-4 tumours than in T1-2 tumours [244].

Brattström	s-VEGF	68	S-VEGF was not correlated to clinical parameters [228].
Takigawa	s-VEGF	125	55 NSCLC, 25 SCLC, 30 benign lung disease. S-VEGF higher in tumour patients compared with healthy
Maniwa	s-VEGF		controls [83]. S-VEGF increases after pulmonary surgery, inducing dormant micro-metastases. AGM-1470 inhibited early recurrence [236].
Matsuyama	s-VEGF	49	S-VEGF correlates with stage and with coagulation-fibrinolysis factors [84].
Kido	s-VEGF	29	20 NSCLC, 9 SCLC. S-VEGF concentration changes in the responders were significantly different from those in the non-responders [86].
Choi	s-VEGF	41	S-VEGF correlates with platelets and leukocytes, not with prognosis [85].
Salgado	s-VEGF		Platelets transports VEGF [245].
Brattström	s-VEGF	58	S-VEGF correlates with tumour volume and
Laack	s-VEGF	118	platelet counts [234]. Low levels of S-VEGF in resected stage I/II patients (n=72) had a longer survival compared with those with
Ueno	s-bFGF	106	elevated levels (>630 ng/L) [235]. 60 NSCLC, 46 SCLC. Prognostic in
Joensuu	s-bFGF	184	SCLC, not in NSCLC [246] 138 NSCLC, 46 SCLC. High bFGF
Brattström	s-bFGF	58	(>3.4 pg/ml) at diagnosis correlates to poorer survival [243]. S-bFGF correlates with recurrent disease, platelet counts and performance
Brattström	s-bFGF	68	status. Independent prognostic factor [234]. Elevated S-bFGF a good prognostic factor in resected NSCLC [228].

Also new targeted therapies with known mediators of angiogenesis in conjunction with standard regimes seem to have prognostic information in selected populations of primary lung carcinomas. Currently, there are 14

ongoing NCI registered anti-angiogenesis studies involving primary NSCLC in various phases [247] and recently the antiangiogenic drug (Bevacizumab), used in a large-scale clinical trial, was shown to prolong survival in metastatic colorectal cancer patients [97]. This was the first drug reported to have significant impact on survival, and now antiangiogenic drugs might be administered to patients with less tumour burden, which would be much more appropriate with regards to tumour biology.

In **Paper III**, three targets (EGFR, HER-2 and COX-2) for newly developed drugs with a putative potential in inhibiting angiogenesis were investigated. The first two targets, EGFR and HER-2, involve signal transduction pathways, whereas the third target, COX-2, is an mitogen-inducible enzyme having anti-inflammatory properties [202], which has been described to stimulate endothelial cell migration and tube formation [205], as described in the introduction. These markers for future targeted therapies were investigated for their role in angiogenesis, determined with a computerised automatic quantification of MVD from CD105 stainings. The automatic MVD calculation is an observer-independent way of minimising bias. However, MVD calculated from CD105 stainings demonstrated no significant correlations with either EGFR or HER-2, but a close correlation, p=0.10, was demonstrated between MVD CD105 and COX-2 positivity. EGFR positivity has earlier been reported to be inversely correlated to angiogenesis determined with Chalkey counting from CD34 stainings [248]. There is more than one possible reason for this contradictory result. Contrary to our study, O´Byrne et al. [248] used CD34 as an endothelial marker and another counting method (Chalkey vs. MVD) and a different cut-off for EGFR positivity (20% vs. 67%). The reason why we chose CD105 as the endothelial marker was based on recent studies demonstrating the superiority of CD105 immunostainings compared with CD34 immunostainings regarding prognostic information [60, 70, 71]. Regarding, HER-2´s correlation

with angiogenesis, recent studies on lung cancer demonstrate also no correlation, as demonstrated in our study [249, 250]. The only significant interrelationship between different markers investigated was seen in tumours having a HER-2+/EGFR- immunohistochemical profile. This molecular biological profile significantly correlated to poorer survival and further subgroup analyses with other prognostic factors. Possible biasing of this finding could not be demonstrated. These findings implicate the future use of Herceptin® as a treatment in NSCLC. However, the HER-family receptors signalling network is complex, as all 4 members (HER-1/EGFR, HER-2-4) can form dimers with each other, homo- and heterodimers and, depending on which isoform is within the signalling complex, the signal might either be enhanced and prolonged or, on the other hand, silenced (HER-3 homodimers) [192]. Further, recent investigations regarding HER-2 inactivation with ZD1839 (Iressa®), Moasser et al. [251] linked the antitumoural effect of ZD1839 (Iressa®) with the Pl3k/AKT pathways. The fact that HER-1 and HER-2, do not have the SH2 domain recognition sequence for the p85 regulatory subunit of Pl3k, whereas HER-3 [252] and HER-4 have [253], also stresses this complexity. So, the adjuvant treatment, recently reported at the ASCO meeting 2003 [14], with surgery followed by chemotherapy, could be the control arm compared with experimental arms including Iressa® and Herceptin® in a future clinical controlled study, depending on the resected tumours HER-2 status.

In **Paper IV**, we further analysed the preoperative material, now aiming for a molecular-biological model predicting recurrences. We demonstrated a significant correlation between endothelial markers CD34 and CD105, however these markers were not correlated with the serum fractions of VEGF or bFGF. No attempt to investigate this correlation has been made in primary lung carcinomas before, but it has been performed regarding advanced ovarian epithelial carcinoma [254]. Barton et al. [254] showed no

correlation between the serum or ascitic concentrations of angiogenic factors (ANG, bFGF and VEGF) and tumour vascularity, which is in conjunction with our data. However, they demonstrated a connection of advanced ovarian epithelial carcinoma and raised serum and ascitic bFGF concentrations with markedly elevated ascitic VEGF was present in most cases. A recent publication from Offersen et al [255], stresses the question about the way microvessel density is estimated in studies, and they further confront the recently published "consensus" [256], regarding Chalkey scoring as the choice for estimating angiogenesis. The authors suggest, based on the observations that the two methods yield counts that are correlated but that the prognostic information from the counts is inconsistent, that future analyses should also focus on measuring angiogenic factors.

Regarding mMVD calculated from CD105 immunostainings, high CD105 mean MVD correlated significantly to high p53 immunohistochemical expression. However, a numerically poorer survival was present in patients with CD105 mean MVD levels above the upper quartile. When CD105 levels were plotted against survival time, in a attempt to explore this finding further, a possible clustering could be demonstrated. High p53 expressions that were correlated to CD105 MVD estimations correlated to better survival, which in turn was inverse to the findings, that demonstrated a numerically poorer survival in the group of patients with high CD105 mean MVD. CD105 is involved in the TGF-β signalling complex, and has been reported to be expressed as two different isoforms. These two isoforms mediate different signals through the TGF-β signalling complex [189], which might be responsible for these discrepancies regarding survival. High p53 expression immunohistochemically is generally believed to be a poor prognostic sign [220], however some authors report the same correlations with survival as we do [220]. So, in an attempt to explain these findings, we went on with a p53 cDNA sequencing study on 32 paired fresh frozen tumour

samples. Although sequencing data correlated with the immunohistochemical data, the immunohistochemical data regarding recurrence rate could not be confirmed. This might probably be due to the small sample size. Another explanation of the survival discrepancies between p53 and MVD calculated from CD105 stainings, might be that p53 mutated tumours trigger the immunosystem earlier, resulting in smaller tumours upon detection and better microscopic metastatic disease eradication. Or, as the complexity in the clinical behaviour of NSCLC tumours is well known, our data might also reflect that the p53 gene or protein aberrations have implications on recurrence rates. This suggests the possibility that p53 gene status and protein expression profile might be of clinical interest when planning treatment. In a recent publication by Bubb et al. [257], it was demonstrated that low expression of p53 in lung primaries was associated with poorer overall survival, and these patients had a higher rate of non-brain distant metastases. Furthermore, patients with brain metastases were particularly prone to develop non-brain distant metastases if the percentage of p53-positive cells in brain metastases was low. Altogether, these data are in conjunction with ours, regarding the predictive impact of p53 immunohistochemical expression. Indications in that direction have also been presented in colorectal and breast carcinomas. In a study by Adell et al. [258], patients with wild-type p53 tumours were seen to experience local recurrence in 27% (13/49) compared with 13% (5/38) in the p53 mutated group without preoperative radiation treatment. With preoperative radiation, the local recurrence rate for p53 negative tumours decreased significantly, whereas the local recurrence rate of p53 positive tumours was unaffected. From the study by Jansson et al. [259] on node-negative breast cancer patients, the inverse finding was demonstrated, as relapses were more frequent in the group of patients with p53 mutations. However, this group of patients gained most from having adjuvant locoregional radiation therapy. In conclusion, the

present study demonstrates that p53 overexpression and CD105 expressions are linked to each other and that p53 overexpression might be indicative of a lower recurrence risk in patients undergoing surgery for NSCLC stage I-IIIA, although future larger prospective studies are needed to fully elucidate this finding.

The quest for prognostic and predictive factors in lung cancer goes on. The new scientific landmarks regarding microchip array techniques and future clinical proteomic applications, raises hope for the future. In contrast to the detailed study of single genes and their proteins, the molecular tumour profiling with large-scale analysis of gene expression by use of DNA microarray technology [260] provides abundant information to explore. DNA microarrays typically consist of rows and rows of oligonucleotide sequence strands or cDNA sequences lined up in dots on a silicon chip or glass slide. Oligonucleotide sequences or cDNAs are used because they can hybridise with mRNA from the tumour sample. Arrays can accommodate more than 20000 specific sequences on a single chip; these sequences can be random or deliberately biased to represent genes that are characteristic of a particular cell type. Gene-expression profiles provide molecular "fingerprints" that are almost exclusive for individual-specific identification of cases [261]. Tumours may be clustered by gene-expression patterns and it is likely that such subgroups comprise distinct subtypes or entities of cancers. In node-negative breast cancers, chip analysis reports different gene-expression signatures separating patients with good and poor prognoses. In the latter group, genes involved in the cell cycle, invasion and metastasis, angiogenesis, and signal transduction were significantly up-regulated [262]. However, even chip arrays have their drawbacks as DNA represents the template, and RNA the interim messenger, but it is the final product (the protein) that actually carries out the effects. Fortunately, proteomics is well on the way to producing a new wave of fascinating data with great potential for the clinician [263-265]. With the use

of the SELDI-TOF (surface enhanced laser disorption ionisation-time of flight) technique, a development of 2D-poly-acrylamide-gel-electrophoresis (2D-PAGE), enabled a distinction to be made between benign and malignant ovarian carcinomas [264]. The authors correctly classified 63 of 66 (95%) of the blinded controls as not cancer and all 50 blinded cancer samples, including all 18 stage I cancers, as malignant. Furthermore, regarding prostate cancer, the same group [266], managed to establish a protein expression profile that correctly predicted 36 of 38 patients with prostate cancer, while 177 of 228 patients were correctly classified as having benign conditions. For men with marginally elevated PSA levels (4–10 ng/mL; n = 137), the specificity was 71%. Altogether, these results point in the direction of high output screening possibilities regarding primary lung cancer.

We have now initiated an investigation of the messengers and the effectors in lung cancer cell lines, where gene-profiles with microchip arrays under different treatment settings, different gene clustering analysis, signalling transduction pathway analysis and expression profiles regarding known markers for chemo- and radiosensitivity are investigated. The SELDI-TOF investigations are in progress and in the first pilot serum study we investigate if we are able to distinguish a specific pattern for NSCLC patients compared with a normal control subject group, consisting of apparently healthy doctors at the Department of Oncology, Uppsala. Data are soon to be published.

Conclusions

1. Angiogenic factors VEGF and bFGF analysed in sera have both predictive and prognostic information when measured in operable NSCLC.

2. The angiogenic factors VEGF and bFGF analysed in sera fluctuate depending upon which therapy and when during the course of treatment the samples are collected in patients with inoperable NSCLC.

3. An elevation of VEGF and bFGF can provide different prognostic information depending upon which treatment (chemotherapy or radiation therapy) that is delivered for patients with inoperable NSCLC.

4. Antibodies directed to inactivate HER-2 signalling might be a future treatment for NSCLC, as the target is overexpressed in a subset of patients with operable NSCLC and that HER-2 positive tumours with a concomitant EGFR negativity, immunohistochemically, have a poorer prognosis in our population of patients.

5. No link between mean MVD and circulating angiogenic factors, VEGF and bFGF, could be demonstrated. However, mean MVD seems to be linked with a p53 immunohistochemical overexpression.

6. In our population of patients with operable NSCLC, p53 expression status predicted recurrent disease and was linked with mean MVD estimated with CD105 immunohistochemical stainings.

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