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The role of HIF (hypoxia inducible factor) in angiogenesis of malignant lung tumours

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Abkürzungsverzeichnis

ABC	avidin-biotin-complex
abl	abelson murine leukaemia viral oncogene homolog
AC	adenocarcinomas
akt	v-akt murine thymoma viral oncogene homolog
Ang	angiopoietin
ARNT	aryl hydrocarbon receptor nuclear translocator
ATP	adenosine triphosphate
BAC	bronchiolo alveolar carcinoma
bax	BCL2 associated x protein
BCL2	B-Cell CLL/lymphoma 2
bcr	breakpoint cluster region
bHLH	basic helix loop
CA	carcinoma
CAIX	carbonic anhydrase 9
CEA	carcino-embryonic antigen
CK	cytokeratin
c-kit	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog; kit oncogene; stem cell factor receptor
COPD	chronic obstructive pulmonary disease
COX2	cyclooxygenase 2
c-src	v-src sarcoma Schmidt-Ruppin A2 viral oncogene homolog
CT	computed tomography
cTGF	connective tissue growth factor
DNA	deoxyribonucleic acid
ECM	extra-cellular matrix
EGFR	epidermal growth factor receptor
ERK	extra-cellular signal-regulated kinase
Fc	fragment crystallisable region
FDA	food and drug association
FGF	fibroblast growth factor

FIH	factor inhibiting HIF
GIST	gastrointestinal stroma tumours
grb	growth factor receptor-bound protein
H&E	hematoxylin and eosin stain
HER2/neu	human epidermal growth factor receptor 2
HGF	hepatocyt growth factor
HIF1 a, HIF1 alpha	hypoxia inducible factor 1 alpha
HRE	hypoxia response element
IARC	International Agency for Research on Cancer
IgG	immunoglobulin G
IHC	immunohistochemistry
IL	interleukine
k-ras	v-ki-ras2 kirsten rat sarcoma viral oncogene homolog
LAB	labelled-avidin-biotin-technique
LC	large cell carcinoma
LCNEC	large cell neuroendocrine carcinoma
LOH	loss of heterozygosity
MAPK	ras-mitogen activated protein kinase
MEK	mitogen activated protein kinase kinase
MMP	matrix metallo proteinase
MMPI	matrix metallo proteinase inhibitor
mRNA	messenger ribonucleic acid
m-Tor	mammalian target of rapamycin
Muc1	mucin 1
MVD	microvessel density
MYC	c-myc myelocytomatosis viral oncogene homolog
NE	neuroendocrine
NO	nitric oxide

NSCLC	non-small cell lung cancer
ODDD	oxygen dependent degradation domain
p16ink4	cyclin dependent kinase inhibitor 2A
PDGFR	platelet derived growth factor receptor
PGE2	prostaglandin E2
PHD	prolyl hydroxylase domain
PI(3)K	phosphatidylinositol triphosphate kinase
PKB	protein kinase B
PKC	protein kinase C
PLCγ	phospholipase C gamma
PlGF	placenta growth factor
PNET	primitive neuroectodermal tumours
pVHL, VHL	von-Hippel-Lindau-protein
raf1	v-raf 1 murine leukaemia viral oncogene homolog
Rb	retinoblastoma protein
RBM	reticular basement membrane
RTK	receptor tyrosin kinase
RTX	radiotherapy, radiation
SCF	stem cell factor
SCLC	small cell lung cancer
SP	surfactant protein
SQCC	squamous cell carcinoma
SRBCT	small round blue cell tumours
STAT	signal transducers and activators of transcription protein
TGF	transforming growth factor
TIE	tyrosin kinase with immunoglobuline-like and EGF-like domains
TK	tyrosine kinase
TKI	tyrosine kinase inhibitor
TMA	tissue micro array
TP (e.g. TP53)	tumour protein

TTF	thyroid transcription factor
VAD	vascular disrupting agent
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

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I. INTRODUCTION

I.1. Summary (german)

Lungenkrebs ist weltweit Todesursache Nummer eins unter den malignen Tumorerkrankungen sowohl bei Männern, als auch Frauen. Die Inzidenz der Lungenkrebsfälle ist weiterhin im Steigen begriffen. Die Fünf-Jahres-Überlebensrate ab Diagnosestellung beträgt mit den derzeitigen Behandlungsmöglichkeiten maximal 15%. Chirurgische Resektion, sofern überhaupt möglich, führt häufig zu Tumorrezidiven und auch Chemotherapieregimes, die den Goldstandard ab Tumorstadium III darstellen, führen zu keiner Überlebensverlängerung.

Die folgende Arbeit beschäftigt sich mit intrazellulären Tumorsignalwegen, die unter Hypoxie aktiviert werden können und den Tumor trotz Sauerstoffmangel zu Wachstum, Gefäßneubildung und Metastasierung veranlassen. Das Ziel war, die beteiligten Signalwege und Tyrosinkinasen (speziell jene dem Hypoxia Inducible Factor, kurz HIF nachgeschalteten Signalwege, die an der Gefäßeinsprossung und –neubildung beteiligt sind) für jeden histologischen Lungenkarzinom-Subtyp zu erfassen, um mögliche neue pharmakologische Ansätze zur Blockade von Tumorwachstum zu finden. Für den Nachweis einer möglichen Präsenz von Hypoxie in Lungentumorgewebe wurde eine immunhistochemische Färbung von HIF1 alpha und Carboanhydrase 9 (CAIX), den sogenannten Hypoxiemarkern, auf Tissue Micro Arrays durchgeführt. Weiters ermittelten wir auch die Expression von TIE (tyrosine kinase with immunoglobulin-like and EGF-like domains) 2, der VEGF-Faktoren (vascular endothelial growth factor) A, B, C und D, sowie ihrer Rezeptoren VEGFR (vascular endothelial growth factor receptor) 2 und 3, und PDGFR (platelet derived growth factor receptor) a und b. Für jede Stanze wurde die Intensität der Färbung und der Prozentsatz der gefärbten Tumorzellen lichtmikroskopisch evaluiert und daraus ein Produkt errechnet. In den statistischen Analysen wurden die einzelnen Faktoren auf ihre Expressionsstärke für jeden histologischen Subtyp untersucht und Präferenzen in der Aktivierung von Signalwegen kamen zum Vorschein.

Weiters wurden Unterschiede in Expressionsmustern der oben genannten Wachstumsfaktoren zwischen NSCLC (non small cell lung cancer)-Subtypen und SCLC (small cell lung cancer) ermittelt und auch die einzelnen NSCLC-Subgruppen wurden gegeneinander verglichen. Am Ende erfolgte ein Vergleich mit den Ergebnissen anderer Forschungsgruppen, die über Literaturrecherche im Pubmed erlangt wurden.

Die folgende Diplomarbeit beginnt mit einer theoretischen Aufarbeitung der Epidemiologie, Ätiologie, genetischen Veränderungen und histologischen Subtypen von Lungenkarzinomen, danach werden die Studienergebnisse präsentiert.

I.2. Abstract

Lung cancer is the leading cause of cancer related death worldwide among both men and women. The incidence of lung cancer is still rising. Five-year-survival-rates from date of diagnosis, using current treatment strategies, are not higher than 15%. Surgical resection is often followed by local relapses and also chemotherapy regimens, which represent the golden standard of treatment for tumour stage III and IV, do not lead to a significant increase in overall survival.

In the following study intracellular tumour signaling pathways were investigated, which may be activated under hypoxia. Hypoxia normally should go along with a shrinkage of the tumour mass, but tumours have found a way to grow and metastasize also in absence of oxygen. The aim was to identify the involved signaling pathways and tyrosine kinases, especially those downstream of hypoxia inducible factor signaling responsible for angiogenesis, in order to develop new pharmaceutical strategies for the blockade of tumour growth mechanisms. To point out hypoxic conditions in lung cancer tissues immunohistochemical staining of HIF1 alpha and carbonic anhydrase 9, the so-called markers of hypoxia, on tissue microarrays was used. Furthermore also expression of TIE2, vascular endothelial growth factors A, B, C and D and their receptors VEGFR2 and 3, and PDGFRa and b were examined. Each core was evaluated for its intensity of staining and the percentage of tumour cells being stained and products were calculated. The results were fed into Microsoft Excel tables and prepared for statistical analysis.

Statistics provided information about preferred expression patterns of angiogenesis growth factors and receptors for each histological subtype of lung cancer. Furthermore, significant differences between NSCLC and SCLC signaling were pointed out.

Also NSCLC subtypes were compared to each other concerning typical activation of angiogenesis pathways. Finally our findings were compared with those of other research groups, which were obtained by literature research in Pub Med.

The following thesis starts with a theoretic part about epidemiology, etiology, genetic changes and histological subtypes of lung cancer. The results of our immunohistochemical study are then presented.

I.3. Epidemiology

Lung cancer is the most common malignancy and the leading cause of cancer death worldwide (1). In 2000 1.1 million people died from lung cancer (2). 53% of these deaths occurred in western industrialized countries, the remaining 47% in the less developed countries (3). The current incidence amounts to 1.2 million new cases per year with the highest rates observed in Europe and Northern America (2).Smoking as a potential risk factor and trigger for the development of lung cancer is found in 90% of these patients (4).

Historically more men than women have died from lung cancer as a result of higher levels of smoking among the male sex. The male : female mortality ratio, however, is getting more and more narrow (3). Lung cancer today is the major cause of cancer related mortality in both men and women in industrialized countries and causes more deaths than colorectal, breast and prostate cancer together (4).

Once diagnosed, the 5-year-survival-rate fluctuates between 8 – 12% in Europe (4). Due to this fact, efforts in earlier diagnosis, earlier therapy and new pharmacological strategies are reasonable.

I.4. Etiology

Smoking is the major cause of lung cancer (2). 85% of the lung carcinomas in men and 47% of the lung carcinomas in women are a consequence of smoking. Therefore a major reduction in tobacco consumption could prevent a large number of lung cancers. Cessation of smoking for over 20 years reduces the risk to that of never-smokers.

Many carcinogens and toxins (n-nitrosamines, polycyclic aromatic hydrocarbons, aromatic amines, aza-arenes, aldehydes, volatile hydrocarbons, nitro compound, heavy metals) causing inflammation are contained in the inhaled smoke. Overall, there are no real differences between filter and non-filter cigarettes, light cigarettes with low tar and low nicotine levels compared to normal cigarettes or cigarettes with black or blond tobacco. In the end they can all lead to the development of a carcinoma. Filterless cigarette smoking results in a higher particle fraction, leading to the development of squamous cell carcinomas or small cell carcinomas. Since the fumes of filter cigarettes are inhaled more deeply, they tend to evoke the formation of adenocarcinomas.

Another identified risk factor for the development of lung cancer is the occupational exposure to agents like asbestos, Radon, polycyclic aromatic hydrocarbons, heavy metals and crystalline silica. According to the IARC (International Agency for Research on Cancer) these substances are proven as potential carcinogens.

I.5. Genetic and molecular alterations

Carcinogenesis of lung cancer occurs in a multi-step fashion (2). There is a stepwise malignant progression of cells through accumulation of several genetic alterations like allelic losses (LOH), chromosomal instability or imbalance, mutations in oncogenes, mutations in tumour suppressor genes and gene silencing through promotor hypermethylation. Differences in the type of genetic alterations and time of appearance of genetic alterations can be found between the different histological subtypes and will be described in the next chapter.

Common allelic losses in lung cancer can be found on the following chromosomes: 3p 14-23, 8q 21-23, 9p 21, 13q, 17q, 18q and 22p.

Inactivation of TP (tumour protein) 53 and its anti-proliferative role can also be found in 50% of NSCLC and 70% of SCLC. Inactivation of Rb (retinoblastoma protein) 1 as gatekeeper for G1 – S transition in the cell cycle is typical for lung cancer as well. And last but not least loss of fragments of chromsome 3p is common in 80% of the NSCLC and SCLC.

I.6. Histological Subtypes

Lung cancer is divided in two main histological groups. Non-small cell lung carcinomas (NSCLC) represent 80% of the overall lung cancer cases and 20% are small cell lung carcinomas (SCLC). The group of NSCLC includes adenocarcinomas (AC), squamous cell carcinomas (SQCC), large cell carcinomas (LC) as well as other infrequently found carcinomas (5). The preferred localization of SQCC is the central part of the lung, whereas AC especially arise in periphery. AC have replaced SQCC as the most frequent NSCLC in industrialized countries (6). The introduction of filter cigarettes and the reduced nicotine content seem to be the main causes for this shift (7).

I.6.1. Squamous cell carcinoma

SQCC represent tumours of malignant epithelial origin, arising from the bronchial epithelium (2). Often they show areas of keratinization. 90% of the SQCC are found among smokers. Besides also arsenic plays a role in the carcinogenesis of SQCC. This sort of tumour is usually found centrally in the main stem, lobar or segmental bronchi.

The tumour cells are rather large with hyperchromatic nuclei and one or more nucleoli and abundant cytoplasm. Besides areas of necrosis, tumour cells can be found in aggregates with elongated or spindle nuclei. Morphologically, SQCC appear white or gray, depending on the rate of fibrosis, and are of firm consistence. They can reach enormous sizes and often cavitate. In the course of tumour growth the bronchial lumen can occlude, followed by stasis of bronchial secretions, atelectasis and obstructive lipoid pneumonia or infective bronchopneumonia. In advanced cases, peripheral SQCC may involve the chest wall or diaphragm directly through the pleura.

Tumours grow intraepithelially (in situ) or with subepithelial invasion and/or endobronchial polypoid growth. Among early invasive SQCC a creeping type with lateral growth along bronchial mucosa, replacing surface epithelium, and submucosal micro invasion and a penetrating type with small polypoid mucosal lesions and downward invasion can be distinguished. Peripheral SQCC grow intrabronchiolar as solid nodules with or without intraepithelial extension.

SQCC grow locally aggressive. Below a diameter of 2 cm there are almost no metastases in lymph nodes.

Metastases in distant organs are not so common in SQCC compared to other histological subtypes of lung cancer. However, poorly differentiated SQCC early metastasize in organs such as the brain, liver, adrenals, lower gastrointestinal tract and lymph nodes.

Depending on the degree of differentiation tumour cells show keratinization, pearl formation and intracellular bridges. Several subtypes of SQCC have been defined:

 Papillary forms with exophytic/endobronchial growth, which go along with invasion in most cases.

 The clear cell variant with cells of clear cytoplasm, which must be distinguished from AC and LC of the lung with an extensive clear cell change and also from metastatic clear cell carcinoma of the kidney.

 The small cell variant, which is poorly differentiated with small cells of typical NSCLC characteristic and focal squamous differentiation. Differential diagnoses are combined SCLC with parts of SQCC and true SCLC. More prominent nucleoli, more cytoplasm, more distinct cell boarders, vesicular chromatin, intercellular bridges and keratinization are indicators for the diagnosis of SQCC.

 The basaloid variant, which is characterized by prominent peripheral palisading of nuclei.

Using immunohistochemistry SQCC express high molecular weight keratin, cytokeratin 5/6 and CEA (carcinoembryonic antigen).

Differential diagnoses of SQCC are LC with squamous differentiation and thymic SQCC as well as a metastasis of a SQCC from another site.

Having a look on molecular genetics of SQCC, mutations are exceedingly rare, whereas protein expression and amplifications are almost similar to AC. HER2/neu (human epidermal growth factor receptor 2) expression and activating mutations of k-ras, for example, are relatively rare in SQCC and more typical for AC. Point mutations in the p53 tumour suppressor gene occur often whereas disruption in the Rb gene pathway is more usual in SCLC. Epigenetic gene silencing via methylation and histone deacetylation is mostly found in tumour suppressor genes. Concerning gene expression profiles, SQCC go along with high-level expression of keratin genes 5, 6, 13, 14, 16, 17 and 19.

Survival rates of patients with SQCC are better than those of patients with AC. This trend can be found among all tumour stages. The 5-year-survival-rate for T1N0M0 is 80%. Extensive necroses go along with worse prognosis. Females have a better overall survival, which is, however, more significant for AC. Generally, the stage of disease and the performance status at diagnosis are the most powerful prognostic indicators.

I.6.2. Small cell lung carcinoma

SCLC are malignant epithelial tumours with cells varying in shape and only small cytoplasm (2). Cell borders are not well defined, the chromatin is dense heterochromatic. Nucleoli are not visible. The shape of nuclei ranges from round over oval to spindle-shaped. The nuclear/cytoplasm ratio is high. Often large areas of necrosis can be found in the tumours. They are located in the central part of the lung and spread loco-regionally, but lymph node, as well as distant metastases, are frequently found on initial diagnosis. Tumour cells form minimal cohesive aggregates, with well apparent nuclei and many mitoses.

Having a look on signs and symptoms, SCLC reflect their central location and loco regional spread with hoarseness and vocal cord paralysis. Often also symptoms of metastases (esp. brain metastases) or paraneoplastic symptoms are present.

In imaging, SCLC present with hilar and/or perihilar masses and also mediastinal lymphadenopathy.

Morphologically, SCLC is white in colour, rather soft with necroses. They grow submucosally or along bronchi and often involve lymphatic vessels. Only 5% can be found in peripheral areas of the lung.

Because SCLC usually present with widespread dissemination at diagnosis, it is staged as limited or extensive disease. To date, however, an application of the TNM-System for SCLC is in discussion.

Using immunohistochemistry, SCLC are positive for neural cell adhesion molecule, chromogranin, synaptophysin and also TTF (thyroid transcription factor) 1 in up to 90% of cases.

Next to classical SCLC also combined forms of SCLC exist, which combines classical SCLC with components of NSCLC (SQCC, AC, LC).

Differential diagnoses of SCLC are lymphoid infiltrates and other neuroendocrine tumours, other SRBCT (small round blue cell tumours), other primary or metastatic NSCLC and carcinoid tumours (less necroses, lower mitotic and apoptotic activity than SCLC). PNET (primitive neuroectodermal tumours) also have fewer mitoses. Especially the differentiation of NSCLC can be difficult and runs best via high quality H&E (hematoxylin and eosin stain**)** sections assessing cell size, nuclear: cytoplasm ratio, chromatin and nucleoli.

On the somatogenetic base, SCLC are invariably aneuploid. Deletions can be found on 3p, 4, 5q, 10q, 13q, 17p and gains on 3q, 5p, 6p, 8q, 17q, 19 and 20q. 3p deletions are present in nearly 100% of cases.

Together with carcinoids and large cell neuroendocrine carcinomas SCLC belong to the neuroendocrine (NE) tumours. SCLC, however, are directly associated with tobacco smoke, whereas carcinoids are not related to smoking habits. When comparing SCLC with NSCLC, MYC (v-myc myelocytomatosis viral oncogene homolog (avian)) amplifications and methylation of caspase 8 are typical for SCLC, whereas mutations in ras and COX2 (cyclooxygenase 2) over-expression are characteristic for NSCLC. The rate of p53 mutations is much higher in SCLC than in any other kind of lung cancer. Furthermore, inactivation of Rb, up-regulation of BCL2 (B-Cell CLL/lymphoma 2) and telomerase, loss of laminin 5 chains, inhibition of MMP (matrix metalloproteinase) and expression of vascular growth factors are common in SCLC.

Frequency and pattern of mutation in lung cancer are strongly related to cigarette smoking; especially guanin-to-thymin transversions are more common in smokers than in never smokers.

Prognosis is worse for extensive stages of disease, poor performance status, elevated serum lactat dehydrogenase and alkaline phosphatase and low plasma albumin.

I.6.3. Adenocarcinoma

AC represent malignant epithelial tumours with glandular differentiation and in many cases also mucin production, growing in acinar, papillary, broncho-alveolar or solid patterns (2). They are the most common malignant lung tumour in smokers and never-smokers. Typically, AC appear as nodules in peripheral areas of the lung. They have surpassed SQCC as the most common histological subtype.

In imaging like radiography and CT (computed tomography), AC represent peripheral nodules < 4cm. Only in a few cases they are found in central location as hilar/perihilar masses and also cavitation is only rarely seen. Pleura and chest wall involvement are found in 15% of AC cases.

Cells lie arranged in morulae, acini, pseudo papillae and true papillae formations or in sheets of cells. Cell boarders usually cannot be seen. They are rich of cyanophilic cytoplasm and often contain mucin filled vacuoles in the cytoplasm, sometimes relegating the nucleus to the fringe. These cells are then the so-called signet ring cells.

Tumour cells of AC usually have one eccentric nucleus round or oval shaped with finely granular chromatin and one prominent nucleolus.

Macroscopic growth patterns include single or multiple peripheral nodules with a wide range of size, which are of gray or white colour, sometimes including central fibrosis, necrosis, and hemorrhage. Central endobronchial tumours grow as plaques and often lead to bronchial luminal obstruction. Diffuse pneumonia-like growth with lobar consolidation, diffuse bilateral widespread nodules with pleura thickening, so called "pseudomesotheliomas" and, last but not least, AC arising in the background of an underlying fibrosis are also possible growth patterns of AC.

Tumours spread over lymphatic and haematogenous routes, but aerogenous dissemination along the airway within the same lobe or in different lobes, ipsilateral or contralateral is also possible, typically for BAC (bronchiolo-alveolar carcinomas). Peripheral AC may spread over pleural surfaces mimicking mesotheliomas. 1/5 of AC has distant metastases at the time of diagnosis, especially in the brain, bone, adrenals and liver.

Histologically, the mixed subtype of AC is most common. It includes different histological subtypes with different degrees of differentiation and cytological atypia.

Major histological subtypes are acinar and papillary adenocarcinomas, which can be well, moderate and poorly differentiated, bronchiolo-alveolar AC, which are always well differentiated, and solid AC with or without mucin production.

 The acinar pattern consists of acini and tubules of cuboidal or columnar cells able to produce mucin.

The papillary pattern is characterized by papillae with secondary and tertiary papillary structures, necroses and lung invasion. The lining cells are cuboidal to columnae shaped, mucinous or non-mucinous.

The BAC (bronchiolo alveolar carcinoma) shows growth of neoplastic cells along pre-existing alveolar structures. No stromal, vascular and pleural invasion is the precondition for diagnosis. Criteria for invasion would be cytological atypia, fibroblastic stromal reactions and an acinar pattern of growth. Septal widening and sclerosis are also rather common. The non-mucinous BAC shows a Clara cell or type II cell differentiation. Clara cells are columnar with cytoplasmic snouts, have a pale eosinophilic cytoplasm and their nuclei are located apical. Type II cells are cuboidal or dome-shaped with fine cytoplasmic vacuoles. Cytoplasm could range from a clear to a foamy type. The mucinous BAC is more aggressive than the non-mucinous BAC. It has tall columnar cells with basal nuclei and a pale cytoplasm with a varying amount of cytoplasmic mucin. Mucin pooling occurs in the surrounding alveolar spaces. There is only minimal atypia. Aerogenous spreading and satellite tumours are common for the mucinous BAC.

The solid AC with mucin production is composed of sheets of polygonal cells, lacking acini, tubules and papillae.

To sum up, AC with mixed histological patterns, which could be all from the above-mentioned, are defined as invasive tumours, where the extent of stromal fibrosis and inflammation varies.

Some other special forms of AC are the fetal AC, where the tumour resembles fetal lung tissue with glandular elements and tubules of glycogen rich non-ciliated cells. The mucinous (colloid) AC, which is similar to the mucinous AC of the gastrointestinal tract, includes pools of mucin-islands around neoplastic epithelium and is typically well differentiated. The mucinous cystadenocarcinoma with a fibrous tissue capsule and a cystic change with mucin pooling, the signet ring AC and last but not least, the clear cell AC are also counted among the subtypes of AC.

In immunohistochemistry, AC are positive for epithelial markers such as anion exchanger 1 and 3, CK (cytokeratin) 8 and CEA.

Differential diagnoses of AC of the lung are metastatic AC, mesotheliomas, atypical adenomatous hyperplasia and reactive pneumocyte atypia. When there is evidence for metastases the patient usually has a history of a primary tumour and it could be helpful to compare slides of the primary tumour with the lung metastasis. Metastases are also more homogenous, whereas AC of the lung typically have a mixed histological pattern. Metastases normally turn up as multiple lesions in the lung. Immunohistochemistry can be also helpful for differentiation, as 60% of pulmonary AC express surfactant protein (SP-A, pro-SP-B, pro SP-C) and 75% express TTF1. Expression of surfactant proteins is lung specific. CK7 and CK 20 can also be helpful for the differentiation of primary versus metastatic AC. Pulmonary AC are CK7 positive and CK20 negative. Mucinous BAC are CK20 positive and TTF1 negative.

Grading is defined by three grades: 1 = well differentiated, 2= moderately differentiated, 3= poorly differentiated. This assessment score is usually used for acinar and papillary AC. BAC are normally grade 1, solid AC grade 3.

Concerning somatic genetics AC are near diploid with simple numerical chromosome changes. Typical are loss of the Y chromosome and gain of autosomes 1 and 7. 1 q over-expression goes along with higher haematogenous dissemination. Deletions on 3p, 4q, 5q, 6q, 8p, 9, 13q and gains on 5p, 8q and 20q are often present.

Among genetic alterations, point mutations in the k-ras oncogen are found in 25% of AC and patients obtaining this mutation are mostly smokers. EGFR mutations can also be found in 25% of AC and mutations of b-raf in 1 – 4% of AC. In almost 50% of AC no specific genetic changes are known. Amplifications take place in less than 10% of AC. But also mutations of TP 53, inactivation of p16Ink4 (cyclin dependent kinase inhibitor 2A) and up-regulation of the p27 expression are quite common. These mutations effect downstream signaling and induce proliferation. HER2neu and COX2 over-expression are also seen in AC.

Concerning prognosis, high grading and poorly differentiated AC tend more often to local recurrences and lymph node metastases. Papillary growth patterns are also associated with a worse prognosis. Of course, also vascular invasion, increased mitosis and extensive necroses go along with poor outcome. The BAC has a 5-year-survival-rate of 100% (precondition of **NO** pleural, vascular or stromal invasion). Limited resection is reasonable for small (<2cm, entirely sectioned) non-invasive tumours and lack of central fibrosis.

I.6.4. Large cell carcinoma/large cell neuroendocrine carcinoma

This kind of carcinoma belongs to the undifferentiated NSCLC, which means it has no SCLC, SQCC or glandular differentiation (2). Large cell carcinomas account for 9% of all lung cancers and predominate in smokers. They appear mostly in peripheral areas of the lung. LCNEC (large cell neuroendocrine carcinomas) make up 3% of all lung tumours. The average age at diagnosis of LC/LCNEC patients is 60 years.

Tumours are accessible by transthoracic fine needle aspiration and bronchoscopy; however, specific diagnosis can only be achieved on surgical material.

Histologically, LC are poorly differentiated and diagnosis is usually made by exclusion. Tumour cells usually form nests of large polyclonal cells but also trabecular growth, rosettes and palisading patterns are rather common, especially among LCNEC. Nuclei are extremely irregular shaped with equally irregular distribution of chromatin. Cell borders are variable. Nucleoli are prominent and medium-sized and the cytoplasm is normally stained basophilic and found in a moderate amount. There is a high nuclear/cytoplasmic ratio. The prominent nucleoli allow a separation of LCNEC from SCLC.

Subtypes of large cell carcinomas are LCNEC with neuroendocrine features, which are distinguished from SCLC by the presence of very prominent nucleoli and larger nuclei. Another one is the basaloid carcinoma with a pattern of individual tumour cells and tumour cells lying in aggregates with nuclear palisading.

Combined LCNEC includes LCNEC differentiation and a component of AC, SQCC, giant cell or spindle cell carcinoma.

The basaloid carcinoma is of solid nodular or trabecular invasive growth and peripheral palisading. Tumour cells are small, monomorphic, cuboidal with hyper-chromatic nuclei, granular chromatin and absent or focal nucleoli. The mitotic rate is high. Hyaline or mucoid degeneration of the stroma is typical. Furthermore, small cystic spaces and comedo type necroses are rather common. Normally, there is no neuroendocrine marker positive in basaloid carcinoma. Cytokeratin expression of CK 1,5,10 and 14 is possible. TTF1 expression is negative.

The lymphoepithelioma-like carcinoma shows syncytical growth patterns, large vesicular nuclei, prominent eosinophilic nucleoli and heavy lymphocytic infiltration. The lymphoid reaction is characterized by mature lymphocytes, plasma cells, histiocytes, neutrophils and sometimes eosinophils.

Clear cell carcinomas consist of large polygonal cells with clear or foamy cytoplasm.

LC with a rhabdoid phenotype must contain at least 10% of rhabdoid cells, which are characterized by eosinophilic cytoplasmic globules. The eosinophilic colour arises from aggregates of intermediate filaments (vimentin).

Morphologically, LC are found as large peripheral tumour masses, easily seen in chest radiographs, which often involve large or sub-segmental bronchi and invade the visceral pleura and chest wall. They appear as rather soft, pink-tanned tumours including necrosis, hemorrhage and cavitation.

Typical locations for metastases are the hilar and mediastinal nodes, the pleura, liver, bones, brain, pericardium and abdominal lymph nodes.

LCNEC, combined LCNEC, LC with rhabdoid phenotype and basaloid carcinoma have a worse prognosis than the classic LC. LCNEC is usually diagnosed in stage III or IV.

Differential diagnoses are poorly differentiated SQCC with foci of keratinization and intracellular bridges, solid type AC, atypical carcinoids and basaloid carcinomas. When comparing LCNEC with atypical carcinoids, the mitotic rate is higher in LCNEC and they also show areas of more extensive necroses.

In somatic genetics, LC are aneuploid neoplasms of a near triploid range or above. Karyotypes are complex, with a high chromosomal instability and the generation of DNA (deoxyribonucleic acid) copy numbers changes frequently.

On the molecular genetic base, there are similar alterations as in other NSCLC, including mutations of k-ras, p53 inactivation, alteration of the Rb pathway with loss of p16INK4 expression and hyper-expression of cyclin D1. LCNEC, in addition, also show an up-regulation of BCL2 and telomerase and a down-regulation of bax (BCL2 associated x protein).

For prognosis performance status at diagnosis and TNM stage are important. Although basaloid LC are often diagnosed in stage I or II they have a worse prognosis than classic LC. Survival for stage I LCNEC is significantly shorter as compared with stage I NSCLC or stage I LC.

I.6.5. Pleomorphic Carcinoma

Pleomorphic carcinomas are poorly differentiated NSCLC, consisting of a SQCC, AC or LC component plus spindle cells and/or giant cells or containing only spindle and/or giant cells (2). The spindle or giant cell element should compromise at least 10% of the tumour. The presence of an AC or SQCC part should be documented specially, whereas foci of LC need not be mentioned.

Mitotically active spindle cells are arranged in a fascicular or storiform growth pattern. Morphological appearances range from epitheloid to mesenchymal, sometimes with occasional smooth muscle features. The stroma may be fibrous or myxoid. Malignant giant cells are polygonal, uni- or multinucleated and have dense eosinophilic cytoplasm and pleomorphic nuclei. Invasion of large vessels and extensive necroses are commonly seen.

I.7. Short introduction in immunohistochemical techniques

The general principle is based on the use of antibodies against antigens. Polyclonal antibodies are produced from different B-lymphocytes and react with different epitopes of the antigen. They are usually raised in rabbits, goats, pigs or horses. Monoclonal antibodies are produced from plasma cell clones and react with only one specific epitope of the antigen. Monoclonal antibodies are mostly raised in mice (8).

B-lymphocytes are isolated from the spleen or lymph nodes of the mouse and are then fused with mice myeloma cells, forming a so-called hybridoma. Antibody producing hybridomas are cultivated and tested for their specific immunogenic reaction. If the reaction is satisfying, hybridomas are increased in culture medium or in the peritoneal cavity of mice (8).

Antibody titers, antibody dilutions, time of incubation and incubation temperature are responsible for the quality of immunohistochemical staining. The antibody titer is the highest dilution of an anti-serum leading to the optimum of specific staining and the least background reaction. The antibody dilution, if not pre-fabricated or specified by the producer, should be determined by dilution series. The time of incubation should be defined before selection of the antibody titer. The higher the titer, the shorter becomes the necessary time of incubation. The equilibrium between antibody/antigen reaction is faster reached at 37° C than at room temperature. When the temperature of incubation is increased, higher dilutions or shorter time of incubation should be chosen (8).

Immunohistochemical staining is based on an enzyme/substrate reaction. Commonly used enzymes are horseradish peroxidase, alkaline phosphatase from calf bowel, glucose oxidase from aspergillus niger and beta galactosidase (8).

Concerning the methods of staining direct and indirect methods are possible.

I.7.1. Direct Method

The enzyme-linked primary antibody reacts with the tissue antigen, followed by the substrate/chromogene reaction. Only one antibody is necessary for the incubation. This method can be quickly and easily conducted causing only little unspecific reactions (8).

I.7.2. Indirect Method 2 step variant

First, an unconjugated primary antibody reacts with the antigen. Then, a second enzyme-linked antibody against the Fc-fragment (fragment crystallizable region) of the primary antibody (which is now acting as antigen) is used. Then, the substrate/chromogene reaction takes place. Several secondary antibodies are reacting with different epitopes of the primary antibody, leading to a shift of many enzyme molecules upon the antigen and, therefore, causing a more intense staining (8).

I.7.3. Indirect Method 3 step variant

In this case a third enzyme-linked antibody against the secondary antibody is used. The secondary and tertiary antibodies are coupled with the same enzyme. The tertiary antibody is brought in for further enhancement of the staining. More enzyme molecules are attached to the tissue antigen leading to a higher intensity of staining. This option especially makes sense when antigens with only a few epitopes should be detected (8).

I.7.4. Avidin-Biotin method

This method is based on the stronger affinity of avidin for biotin. Two subtypes of this kind of staining are common: the ABC (avidin, biotin complex method) and the LAB (labeled avidin biotin technique). A biotinolyted secondary antibody is necessary in both cases. Free binding sites on avidin from the avidin/biotin complex or from enzyme-linked avidin bind to the biotin of the bridge antibody. The order of application of reagents runs as follows: primary antibody, biotinolyted secondary antibody, pre-formed avidin-biotin enzyme complex or enzyme-labeled avidin and substrate solution (8).

Horseradish peroxidase or alkaline phosphatase are typically used as enzymes. The avidin/biotin method has the highest sensitivity of all staining methods according to the very strong affinity of avidin for biotin (8).

I.8. Receptor tyrosine kinases and their intracellular signaling as potential new targets in anti-tumour therapies

Interaction of receptor tyrosine kinases (RTK) and binding of their growth factors, also called ligands, holds the central role in the process of angiogenesis. The activated receptors form homodimers or heterodimers, and furtheron activate the intracellular kinase domain via phosphorylation. This action is followed by activation of their downstream signaling cascades that stimulate proliferation and survival. Two major intracellular signaling cascades are activated by RTK: ras-mitogen-activated-protein-kinase (MAPK) and phosphatidylinositol-3-OH-kinase (PI(3)K)-Akt (v-akt murine thymoma viral oncogene homolog)-mTOR (mammalian target of rapamycin). The entire collection of kinases encoded by the human genome includes >500 protein kinases which represent a rich and diverse source of potentially drug targets for disrupting tumour growth and survival (9).

TK (tyrosine kinases) are enzymes that transfer γ-phosphate groups from ATP (adenosine triphosphate) to the hydroxyl group of tyrosine residues on signal transduction molecules. Phosphorylation of signal transduction molecules is a major activating event that leads to dramatic changes in tumour growth. Some TKs, such as EGFR-TK, can autophosphorylate when activated, as well as phosphorylate other signaling molecules. Tyrosine kinases play a central role in signal transduction, acting as relay points for a complex network of interdependent signaling molecules that ultimately affect gene transcription within the nucleus. Strict regulation of TK activity controls the most fundamental processes of cells such as the cell cycle, proliferation, differentiation, motility and cell death or survival. TK receptors transduce signals from both outside and inside the cell. The non-receptor TKs are found in the cytoplasm; they lack a transmembrane segment and generally function downstream of receptor TKs. The bcr (breakpoint cluster region)-abl (abelson murine leukaemia viral oncogene homolog) fusion protein and c-Src (v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog) are examples for non-receptor TKs that transduce signals inside the cell (10).

Clinical agents to inhibit the activity of these molecules have been developed only for a few of these TK. Examples of clinically targeted transmembrane receptor TKs include the EGFR, HER-2, PDGFR, VEGFR and c-kit (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT)) receptor. These new targeted therapies are designed to take advantage of the molecular differences specific to tumour cells compared with normal tissues. The goal is to achieve better tumour responses with better safety profiles than those associated with cytotoxic chemotherapies. In the case of EGFR-TK oncogenic activation can, for example, occur by several mechanisms: excess ligand expression or high expression of EGFR, activating mutation, failure of inactivation mechanisms or transactivation by receptor dimerization. EGFR-TK activity plays a key-role in numerous processes that affect tumour growth and progression, including proliferation, dedifferentiation, inhibition of apoptosis, and metastasis (through effects on cell migration, invasiveness and lack of adhesion dependence). Activity of EGFR-TK also influences tumour angiogenesis by up-regulating expression of VEGF and IL-8 (interleukine 8) (10).

Tyrosine kinase inhibitors such as gefitinib, sorafenib etc. are different from antibody-based therapies as they enter tumour cells and directly interfere with TK enzymes that are aberrantly activated in tumour cells and are critical for the growth of the tumour. Targeting these RTK with therapeutic antibodies to RTK ligands or receptors themselves or small molecule inhibitors that target intracellular kinase domains of the RTK seem to be promising new possibilities in anticancer therapy (9).

I.9. VEGF pathway

This pathway plays an essential role in the formation of blood vessels (see figure 1), which could either occur by differentiation of endothelial precursor cells (angioblasts) in situ, which then form primitive vessels = vasculogenesis, or by growth of pre-existing vessels = angiogenesis. Vasculogenesis leads to development of the primary vascular plexus of the embryo, whereas angiogenesis becomes important in late embryogenesis and adult life (11, 12).

Endothelial cells respond to a variety of extra cellular signals that activate receptors responsible for growth and differentiation. VEGF, Angiopoietin and Ephrin are key molecules in the promotion of angiogenesis via activation of the VEGFR, TIE and Ephrin receptor (13, 14). VEGF signaling is essential in the earliest stages promoting proliferation and differentiation of endothelial lineages. Angiopoietin/TIE2 turn up later for recruitment of supporting cells and vessel stabilization (15). VEGF is a heparin-binding homodimeric glycoprotein that acts via endothelial-specific receptor tyrosine kinases, VEGFR1 (Flt1), VEGFR2 (KDR/Flk1) and VEGFR3 (Flt4) (13).

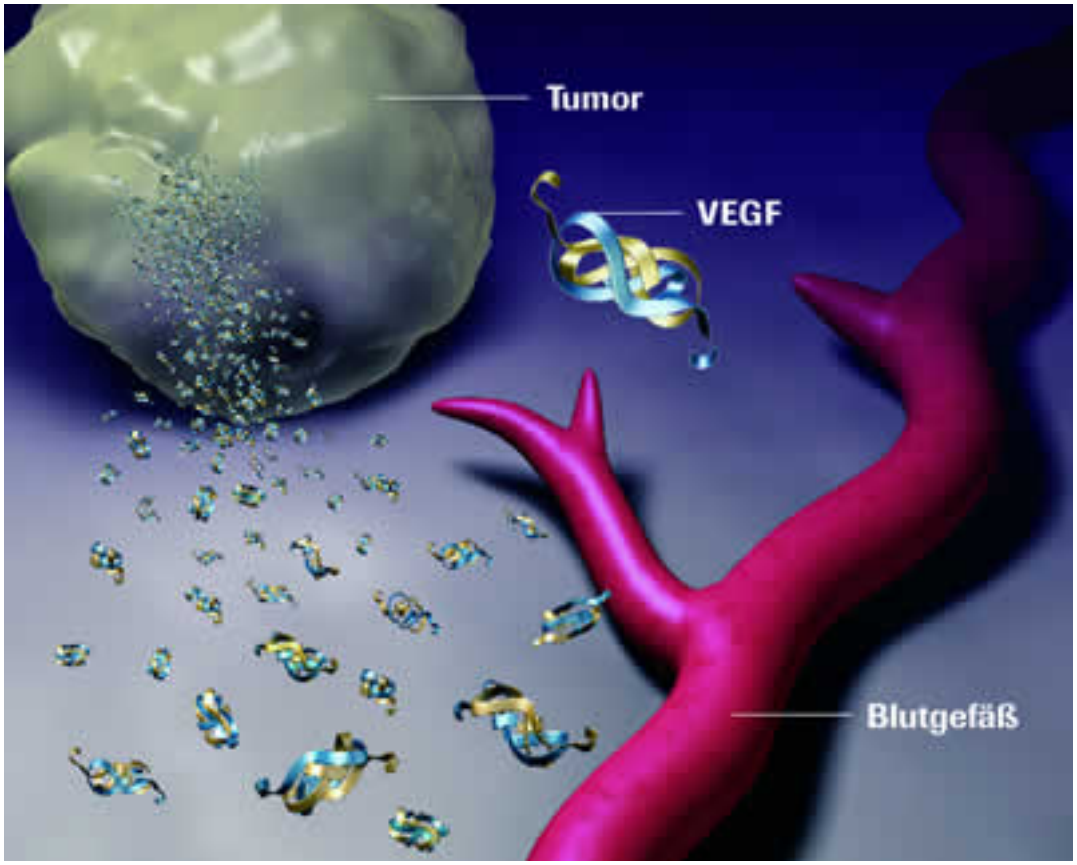


Figure 1: Tumour cells produce VEGF, which induces blood vessel growth towards the tumour in order to maintain supply of nutrients and oxygen for further tumour growth.
http://www.roche.de/pharma/indikation/onkologie/darmkrebs/images/angiogenese.jpg

Besides VEGFA, also PlGF1 and 2 (placenta growth factor), VEGFB, VEGFC, VEGFD and VEGFE belong to the VEGF family. VEGFR2 is the main signal transducing VEGF receptor for angiogenesis and mitogenesis of endothelial cells. VEGF acts primarily through VEGFR2, while VEGFR1 may serve as regulatory receptor. VEGFR3 is predominantly expressed on lymphatic endothelial cells and VEGFC/VEGFD primarily work as its ligands (16).

So this receptor/ligand complex is linked to tumour lymphangiogenesis and lymphatic metastasis (16). After receptor dimerization and autophosphorylation PLCγ (phospholipase C) and PI3K pathway gets activated. Activation of PKC (protein kinase C) plays a crucial role in VEGFA mitogenic signaling via the Raf1 (v-raf 1 murine leukaemia viral oncogene homolog) –MEK(mitogen-activated proteine kinase kinase)-ERK (extracellular signal-regulated kinases) pathway. Cell survival signal is mainly mediated through PI3K-mediated activation of Akt/PKB. VEGFA induces expression of antiapoptotic protein BCL2 (15).VEGF is an important growth factor for therapeutic angiogenesis and vascularization in ischemic limb and myocardium as it increases proliferation and permeability of capillary endothelial cells. However, it has also unwanted side effects like tumour angiogenesis, vascular leakage, edema and inflammation (17). Angiogenesis is a critical process in tumour progression that does not only provide the growing tumour with required oxygen, nutrients and growth factors but also offers the circulatory access that allows tumour cells to metastasize (18). Initiation of angiogenesis is believed to be reliant on an angiogenic "switch" which leads to a complex series of events, starting with the release of tumour-related proangiogenic factors, endothelial cell activation and the release of proteolytic enzymes followed by endothelial cell migration, proliferation and capillary tube formation (19).

In NSCLC VEGF plays an important role in angiogenesis, growth of primary tumour and development of metastases. Elevated levels of VEGF in NSCLC tissue samples are indicators for negative prognosis. Therefore, VEGF seems to be a worthy target for novel therapies. Tumour growth is angiogenesis dependent and neovascularization is required for tumour growth over 2mm. Other factors involved in the VEGF mediated angiogenic process are aFGF (fibroblast growth factor), bFGF, TGFα (transforming growth factor), IL8, angiostatin and prolactin. VEGF is essential for mediating permeability of tumour blood vessels and also has a mitogenic effect. It is a highly potent and specific mediator of angiogenesis. Its mRNA (messenger ribonucleic acid) is up-regulated in most tumour types and, therefore, an attractive option for drug inhibition. Tumour associated vessels are more tortuous, dilated and permeable. This effect decreases drug delivery, leads to hypoxia and, therefore, also the radiation effect decreases. Several strategies of blocking VEGF/VEGF receptor pathways have been evaluated. Particularly, monoclonal antibodies to VEGF and inhibitors of receptor tyrosine kinases are the most widely studied (16). A detailed discussion on anti-angiogenic drugs is following in a separate chapter.

Concerning the relation between the VEGF and HIF pathway a group of researchers found out that imatinib, an inhibitor of c-kit, also decreased HIF1 alpha activity and VEGF expression in SCLC cells. Stem cell factor, the ligand for c-kit, has a direct effect on VEGF expression. This was tested on H526 SCLC cell line. SCF treatment doubled VEGF mRNA expression and VEGF secretion in absence of other exogenous growth factors. Increase of mRNA especially occurred within the first two hours after SCF stimulation and led to no alteration in mRNA stability. VEGF expression is regulated by two general types of stimuli: Oxygen tension and polypeptide growth factors or cytokines as well as oncogenically activated components of their downstream signaling pathways. Low oxygen tension regulates VEGF expression by enhancing transcription of the VEGF gene and by stabilization of its mRNA. Transcription factor HIF1 alpha is the major regulator of VEGF transcription in response to hypoxia and also leads to a prolongation of VEGF mRNA half-life. EGFR, heregulin, insulin, insulin like growth factor I and interleukin 1 β enhance expression of HIF1 alpha protein and its DNA binding capability and, furthermore, also induce increased VEGF expression. This results in normoxic conditions and can increase in hypoxic conditions. PI3K-Akt-mTor signaling enhances HIF1 alpha mRNA translation. MAP kinase pathways correlate with phosphorylation of HIF1 alpha and enhancement of its transactivation function. SCF/c-kit receptor complex is responsible for regulation of angiogenesis in haematopoietic/non haematopoietic malignancies. Imatinib (Gleevec®), a specific inhibitor of c-kit and PDGFR resulted in decreased VEGF expression. Furthermore, multi-targeted kinase inhibitors against c-kit/VEGFR also decreased MVD (microvessel density). SCF enhanced nuclear HIF1 alpha levels, which correlated well with increased HIF1 alpha binding to a consensus hypoxia responsive element. Therefore, SCF in the second line also enhances VEGF secretion and mRNA expression. VEGF expression doubled in c-kit expressing cells treated with SCF relative to controls. Imatinib completely blocked the increase in VEGF confirming that this phenomenon is mediated by c-kit activation. Other targets of imatinib (PDGFR) are not expressed or activated by SCF in SCLC cells. The increase in VEGF mRNA on SCF treatment is transcriptionally mediated. SCF enhances VEGF expression via its effects on the VEGF promotor predominantly through the HIF 1 binding site. However, significant stimulation of VEGF promoter also occurred in the absence of functional hypoxia responsive elements, which leads to the conclusion that c-kit activation may also enhance VEGF promoter activity through non-HIF1 alpha dependent mechanisms (18).

Besides imatinib, also PI3K inhibitors led to a nearly complete inhibition of nuclear HIF1 alpha accumulation, whereas treatment with MAP (mitogen-activated protein) kinase inhibitors reduced increase in nuclear HIF1 alpha by only about a half. Finally, the study showed that micro vessel density and VEGF expression in SCLC correlate well with both stage and prognosis (18).

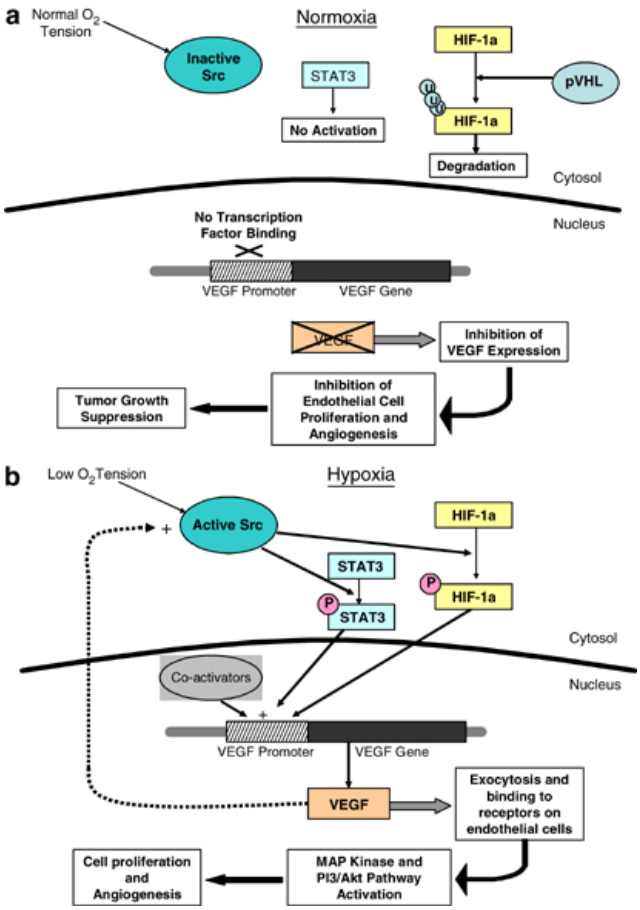


Figure 2: Behavior of HIF1 alpha and STAT3 (signal transducers and activators of transcription protein) under normoxia (a) and hypoxia (b) and their impact on VEGF expression.

http://www.nature.com/bjp/journal/v147/n2s/images/0706635f1.jpg

I.10. HIF1 alpha

Hypoxia inducible factor 1 is a heterodimer consisting of an alpha and a beta subunit, which merge in hypoxic conditions and then act as an active transcriptional factor. Three isoforms of the alpha subunit have been identified: HIF1 alpha, HIF2 alpha and HIF3 alpha. HIF1 alpha is the best characterized and usually forms the heterodimer with the HIF1 beta subunit. HIF2 alpha and HIF3 alpha compete for binding to HIF1 beta. These proteins all belong to the basic helix loop (bHLH) - PER-ARNT-SIM (PAS) domain family of proteins. The beta subunit, also known as ARNT (aryl hydrocarbon receptor nuclear translocator), is constantly expressed (also in normoxic environments), whereas the alpha subunit is up-regulated only in hypoxia. In normoxic conditions (normal atmospheric oxygen concentration of 21%) the alpha subunit is quickly degraded in proteasomes via binding of the von-hippel-lindau protein and ubiquitin within 5 minutes (see figure 2) (20, 21).

Besides hypoxia, however, the alpha subunit seems to be activated also independently from tissue oxygenation via EGFR and its downstream phosphoinositole-triphosphate and MAP kinases (21).

Within the nucleus, the fused HIF binds to the promoter region, the so-called hypoxia-response-element, shortly HRE, and starts transcription of its target genes which regulate processes for cell survival such as angiogenesis (see figure 2), vasodilatation, erythropoiesis, anaerobic metabolism and production of growth factors (20, 21). Target structures in the downstream of HIF are carbonic anhydrase IX and glucose transporter which are nowadays also used as potential surrogate markers of hypoxia (22). We used an antibody against CAIX in our study to outline severe hypoxic conditions in malignant lung tumours. Furthermore HIF is also associated with the expression of VEGF (see figure 2), PDGF and FGF (23).

The proteolysis stability and transcriptional activity of HIF1 alpha are regulated via oxygen dependent hydroxylation of specific amino acid residues via 2 oxoglutarate dependent oxygenases (20, 21, 24).

Hydroxylation of prolyl residues PRO 402 / 564 located in the ODDD (oxygen dependent degradation domain) leads to interaction with the von-Hippel-Lindau E3 ubiquitin ligase complex followed by degradation in proteasomes. There are three prolylhydroxylase domains PHD 1 – 3 (see figure 3) (20, 21, 24).

Hydroxylation of the asparaginyl residue Asn 803 runs via HIF asparaginyl hydroxylase, also known as FIH (factor inhibiting HIF). It blocks the interaction of HIF1 alpha c-terminal domain with transcription co-activators like p300/CBP. However, this process can only take place in the presence of molecular oxygen (21).

The iron II+ 2 oxoglutarate dependent dioxygenases need molecular oxygen. Under hypoxic conditions their hydroxylase activities are decreased and HIF1 alpha can, therefore, not be degraded via the VHL/Ubiquin complex, as non hydroxylated HIF1 alpha shows a very low affinity to the VBC complex (see figure 3) (20, 21).

The formation of the HIF1 alpha/HIF1 beta heterodimer takes place in the nucleus (see figure 3). The fusion with ARNT provides the necessary DNA binding ability to the HREs of the target genes (see figure 3) (20).

The degradation of HIF1 alpha via pVHL is blocked by nuclear sequestration of pVHL in hypoxia or acidosis (24).

The control of HIF levels within the different cell compartments runs via PHDs. PHD 1 is located in the nucleus, PHD 2 in the cytoplasm and PHD 3 can be found in both nuclear and cytoplasmic localization (24).

Phosphorylation acts as an enhancer of transactivation. Direct phosphorylation occurs after stabilization of HIF1 alpha protein in normoxic or hypoxic conditions via p42/p44 mitogen activated protein kinase. There is no effect on the stability or DNA binding functions, however, transcriptional activity of HIF1 alpha is increased as HIF1 beta prefers binding to phosphorylated HIF1 alpha (20). Dephosporylated HIF1 alpha seems to stabilize p53 and induces apoptosis (22).

To sum up, posttranslational modifications like hydroxylation, polyubiquination and phosphorylation etc. regulate the cellular localization, protein/protein interactions, protein stability, DNA binding and transcriptional activity of transcription factors like HIF1 alpha (20, 21, 24).

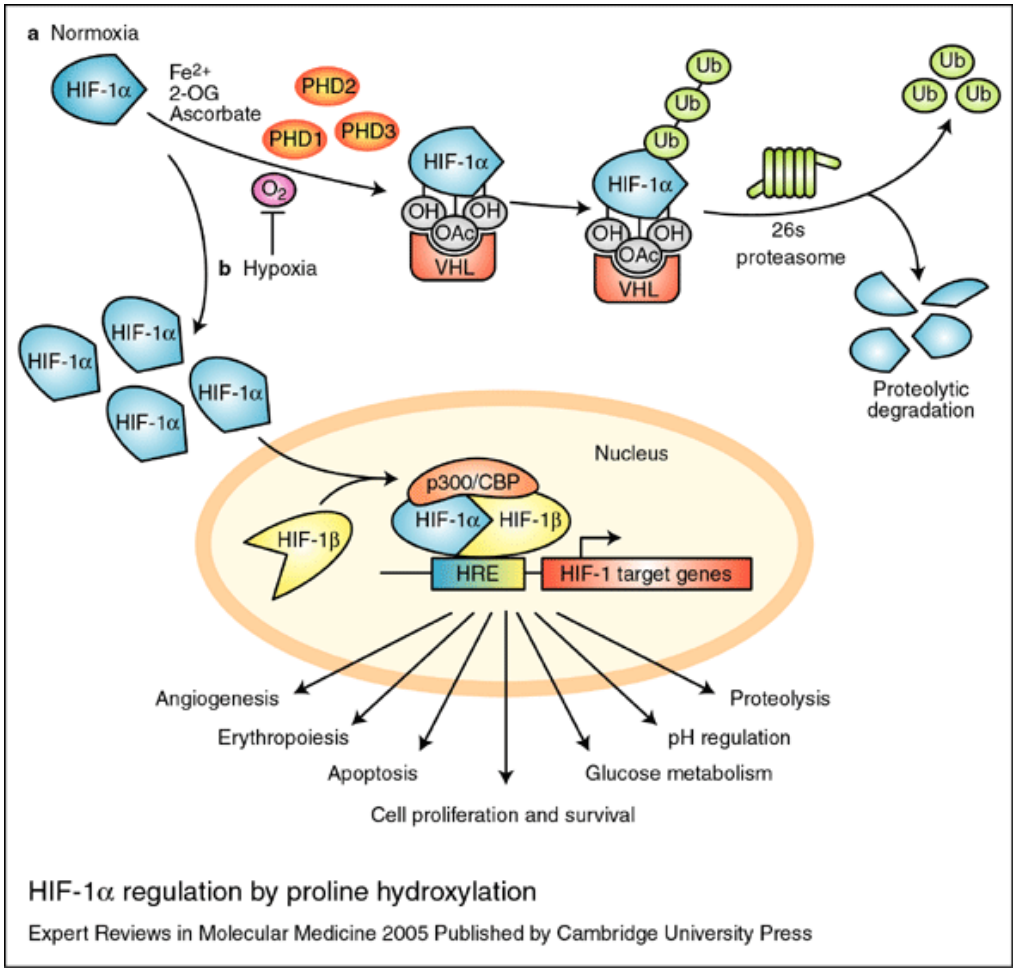


Figure 3: HIF1 alpha regulation by proline hydroxylation
http://www-ermm.cbcu.cam.ac.uk/fig003mas.gif

I.11. CAIX

Carbonic anhydrase IX is a transmembrane enzyme with an extra cellular active site. It is responsible for the reversible metabolism of carbon dioxide to carbonic acid and is, therefore, also responsible for the acidification of tumour environment (25).

It is induced constitutively in some tumour types such as clear cell renal carcinoma. Mutation of VHL protein seems to be responsible for constitutive expression of CAIX. Apart from constitutive expression, CAIX expression is also induced by hypoxia. The HIF pathway is up-regulated by hypoxia or VHL mutations and seems to be the major pathway for CAIX up-regulation in human carcinomas. So CAIX can be regarded as a downstream product in HIF signaling. Especially its strongly perinecrotic localization confirms up-regulation of CAIX under severely hypoxic (= necrosis-inducing) conditions. Hypoxia and acidic pH are linked to aggressive tumour behavior and to resistance of cancer to radiotherapy and chemotherapy. Therefore, CAIX may be useful in oncology as prognostic and predictive marker. CAIX is rarely expressed in normal tissues. Different intensities of hypoxia are necessary for the induction of proteins downstream from HIF activation. Mild hypoxia may be enough for induction of HIF and VEGF whereas CAIX is induced only in severely hypoxic regions. Duration of hypoxia is likely to relate to the following patterns: CAIX positivity patterns stand for chronic hypoxia whereas HIF with a half-life of only a few minutes reflects a state of acute hypoxia. Successful induction of angiogenesis after up-regulation of HIF and VEGF by mild hypoxia may prevent formation of necroses and CAIX induction. Failure of VEGF mediated angiogenesis (e.g. presence of endogenous angiogenesis inhibitors such as angiostatin, irregular perfusion of newly formed vessels) lead to a focal establishment of higher levels of hypoxia followed by CAIX induction (25).

I.12. PDGF pathway

PDGF is a chemotactic growth factor responsible for cell communication with adjacent cells and ECM (extracellular matrix components). These activities form the base for cell migration, angiogenesis and metastasis. It belongs to a family of growth factors including VEGF, PlGF (placental growth factor) and CTGF (connective tissue growth factor). The associated receptors for PDGF are PDGF receptor alpha and beta, which are embedded in membrane invaginations involved in endocytosis. PDGF binding to PDGFR causes internalisation of the ligand/receptor complex into the endosomes. Fusion of endosomes with lysosomes leads to degradation of the receptor/ligand complex, but it can also be degraded in the cytoplasm or in proteasomes (26-28).

The biologically active form of PDGF exists as dimers of polypeptide chains denoted A, B, C and D. Ligand binding to either two of the PDGF receptors (PDGFR) alpha or beta results in receptor dimerisation. Depending on which PDGF dimer, i.e. PDGF-AA, -BB, -AB, -CC and –DD that binds to the receptor, homodimers (alpha-alpha and beta-beta) or a heterodimer (alpha-beta) will be formed (http://www.licr.uu.se/groups/PDGF/index.html).

PDGFR alpha binds PDGFAA and PDGFBB whereas PDGFR beta only binds PDGFBB (29).

Upon ligand binding PDGFR dimerises and autophosphorylates a number of tyrosine residues. Downstream activation of PLCγ, PI3K and ras mitogen activated protein kinase is typical for this pathway (29).

PDGF is produced in a large number of cells, not only in platelets. It is involved in autocrine/paracrine growth stimulation of human tumours such as glioblastoma, melanoma, breast cancer and lung cancer. It is also implicated in the pathogenesis of several non-malignant proliferative diseases including hypertrophic scars, scleroderma of the skin, lung fibrosis, bronchiolitis obliterans or Histiocytosis X. PDGFA expression is essential for the regulation of the female reproductive tract, increasing uterine smooth muscle cells during physiological hypertrophy or pregnancy. PDGFB is involved in the therapy of decubitus ulcers in cases of rather weak wound healing (diabetics!) (27, 28).

I.13. Angiopoietin – TIE2 pathway

Angiopoietins are a new family of growth factor ligands that bind specifically to TIE1/Tek receptor tyrosine kinase. To date four angiopoietins (Ang 1 – Ang 4) bind TIE1 and behave as agonists (Ang 1 and Ang 4) or as context-dependent antagonists (Ang 2 and Ang 3) of TIE1/Tek kinase activity (30).

Angiopoietin regulates two pathways that mediate cell motility, which are PI3K and ras MAPK signaling (30). GRB (growth factor receptor bound protein) 2, GRB 14, GRB 7 and SHP2 bind to TIE2 receptor and also lead to stimulation of PI3K signaling and downstream activation of Akt and PKB pathways and up-regulation of caspase 9. All these processes serve the initiation and potentiation of cell migration (31).

In contrast to Ang 1, Ang 2 does not lead to activation of TIE2 receptor and appears as a naturally occurring antagonist of TIE2 receptor (31). Ang 2 makes mature vessels unstable by blocking the effects of Ang 1 and this destabilization of vessels make them hypersensitive for other types of angiogenic factors (32).

Angiogenesis generally plays a role in processes like embryonic development, wound healing, tumour growth and metastasis, diseases like proliferative retinopathy, post-ischaemic vascularization of the myocardium etc. (31). In case of cancer the idea of blocking aberrant angiogenesis to prevent disease progression seem to be an interesting therapeutic possibility (32).

To sum up Angiopoietin 1 is involved in induction of endothelial cell sprouting, blood vessel maturation during angiogenesis and inhibition of leakage from adult micro vessels via TIE2 receptor (33). Angiopoietin 2 as a natural antagonist, destabilizes vessels and induces neo-vascularization via the VEGF pathway (34).

I.14. Anti-angiogenic therapy in NSCLC

The current standard palliative treatment for patients with NSCLC and a good performance status is double agent cytotoxic chemotherapy consisting of a platinum combined with a third generation agent such as gemcitabine, vinorelbine or a taxane. These agents achieved a palliation of symptoms, improved quality of life, an increase in life expectancy from median of 4 – 5 months to 8 – 10 months and a corresponding increase in the 1-year-survival rate from 10% to 30 – 40%. However, a therapeutic plateau has now been reached with common cytotoxic therapies. The focus is based now on novel target therapies especially on anti-vascular drugs (35).

VEGF seems to be the key cytokine for endothelial sprouting; however other positive regulators such as PDGF, bFGF, MMPs, PlGF, IL 8, HGF (hepatocytes growth factor) and angiopoietins are also involved in the process of angiogenesis. Although the HIF-vHL protein system has a major role in the regulation of VEGF expression, a variety of oncogenes can also enhance VEGF production, including activated EGFR, mutant ras and erbB-2/Her2 (35).

Among anti-vascular therapies angiosuppressive (anti-angiogenic) and vascular disrupting agents (VADs) can be distinguished (35).

I.14.1. Anti-angiogenic therapy

I.14.1.1. Matrix Metallo Proteinase Inhibitors (MMPIs)

In a large randomized phase III study with 774 patients using BMS-275291, a MMPI, combined with carboplatin/paclitaxel chemotherapy compared to chemotherapy alone, there was no benefit from the addition of this agent. Furthermore side effects like increased rates of hypersensitivity reactions, rash and febrile neutropenia can be observed (35).

I.14.1.2. Bevacizumab (Avastin®)

It is a humanized monoclonal antibody that acts via binding and neutralizing VEGF A ligand. It has been licensed as first anti-angiogenic drug for treatment of NSCLC. Used as monotherapy in a phase I trial, bevacizumab showed no response. Combined with cytotoxic therapy a synergistic effect of bevacizumab in progression free and overall survival was reported. In a randomized phase III study of the ECOG (eastern cooperative oncology group), called E4599, they compared chemotherapy with paclitaxel and carboplatin alone vs. paclitaxel, carboplatin and bevacizumab. Addition of bevacizumab was associated with a significant improvement in the median overall survival compared to chemotherapy alone (12.3 vs. 10.3 months). Progression free survival was also significantly improved (6.2 vs. 4.5.months). They also had higher response rates in the bevacizumab arm (35% vs. 15%). Treatment with bevacizumab however, was associated with higher rates of toxicities. There was a significant higher frequency for neutropenia, thrombocytopenia and febrile neutropenia. Aside from haematological toxicity there was also a significant higher rate of hypertension, hemorrhage and proteinuria. As a result of this study the US FDA approved the use of bevacizumab in combination with carboplatin and paclitaxel for the initial systemic treatment of patients with unresectable, locally advanced, recurrent or metastatic, non-squamous NSCLC (16, 35, 36).

This treatment has become the standard of care for these patients in the US. The clinical use of Avastin® is restricted to patients with non squamous histology and those without central nervous system metastases (16, 35, 36).

I.14.1.3. Bevacizumab and EGFR inhibiting agents

It is well known that VEGF and EGFR signaling is interrelated, following similar downstream cascades. One pathway therefore can reduce the activity of the other. VEGF is for example down-regulated by anti-EGFR drugs via HIF1 alpha dependent and independent mechanisms. Anti-EGFR and anti-VEGF therapy has synergistic anticancer effects (16, 35, 36). In a phase II randomized trial with pre-treated non-squamous NSCLC patients three arms (bevacizumab plus chemotherapy, bevacizumab plus erlotinib and chemotherapy alone) had been compared. Bevacizumab and chemotherapy lead to a 34% risk reduction of disease progression or death compared to the other two groups, which both showed a risk reduction of 28%. Progression free survival was 4.8 months in the bevacizumab and chemotherapy arm, 4.4 months in the bevacizumab and erlotinib arm and 3 months in the chemotherapy alone arm. The 6 months overall survival was 62% in the chemotherapy arm, 72% in the bevacizumab and chemotherapy arm and 78% in the bevacizumab and erlotinib arm. However, none of these results was of statistical significance (16, 35). In a phase II study of advanced NSCLC patients chemotherapy with docetaxel or pemetrexed was compared with chemotherapy plus bevacizumab and bevacizumab plus erlotinib. Overall survival was 8.6 months in the chemotherapy arm, 12.6 month in the chemotherapy plus bevacizumab arm and 13.7 months in the bevacizumab plus erlotinib arm. Also these results were not statistically significant (36).

I.14.1.4. Cox 2 Inhibition

The activity of COX 2 leads to the production of prostaglandins, which are also involved in angiogenesis. It was shown that NSCLC are characterized by high values of COX 2 and prostaglandins. High values of COX 2 mRNA and COX 2 expression in NSCLC were associated with poor survival. In a neodadjuvant phase II trial with 29 patients from IB – IIIA NSCLC (see table 1) were treated with celecoxib (COX2 inhibitor) plus 2 cycles of paclitaxel and carboplatin chemotherapy (36).

The overall response rate was 65%. Furthermore, when resecting these tumours, it came out that levels of PGE2 (prostaglandin E2) were much lower in cancer and adjacent lung tissue compared to a control arm (36).

I.14.1.5. Vandetanib (Zactima®)

Vandetanib is a potent small molecule inhibitor of the tyrosine kinase domain of VEGFR2 and has also moderate anti-EGFR activity. Furthermore it inhibits Ret kinase. In pre-clinical studies it led to an inhibition of tumour angiogenesis, growth and metastasis across several tumour types including lung, prostate, colon and breast. In a phase I trial the safety and tolerability of vandetanib was evaluated. An oral dose below 300mg daily is tolerable. Typical adverse events were: rash, diarrhea and an asymptomatic QT-prolongation. Phase III trials comparing chemotherapy plus placebo vs. chemotherapy plus vandetanib 100mg vs. chemotherapy plus vandetanib 300mg have been conducted. The highest median progression free survival was achieved in the chemotherapy plus vandetanib 100mg arm and was 18.7 weeks. There was no statistically significant difference in median overall survival among the three arms. One advantage of vandetanib compared to bevacizumab is the better tolerability among patients with squamous cell histology or brain metastases as none of the patients included in the study suffered from fatal haemoptysis or fatal intracranial bleedings (35, 36).

I.14.1.6. Aflibercept (VEGF Trap, subcutaneous or intravenous injection)

VEGF Trap is a soluble receptor made from the extracellular domains of VEGFR1 and VEGFR2 fused to the Fc portion of human IgG (immunoglobulin G). It binds all isoforms of VEGF and PlGF. Toxicities of phase I trials were hypertension and proteinuria (16, 35).

I.14.1.7. Sorafenib (Nexavar®)

Sorafenib is an oral multi-targeted TKI that inhibits the kinase activity of both C-RAF and B-RAF, VEGFR2 and VEGFR3, PGDGFRb and kit. It has shown benefits in the treatment of renal cell cancer (16, 35, 36).

It was thought particularly active in NSCLC because the proliferation signaling of the Ras/Raf/MEK/ERK pathway is increased in NSCLC due to an increase in K-ras mutations. In a larger phase II trial 52 patients with predominantly stage IV NSCLC were treated with sorafenib. No partial responses were noted. A phase III trial "ESCAPE" (evaluation of sorafenib, carboplatin and paclitaxel efficacy in NSCLC) was stopped early after an interim analysis that demonstrated that the primary endpoint of an improved overall survival would not be achieved. There were more deaths among squamous cell carcinoma patients in the Nexavar plus carboplatin and paclitaxel group than under chemotherapy alone. A phase II ECOG trial of sorafenib in patients with NSCLC who have been treated with two or more prior chemotherapy regimens is in progress (16, 35, 36).

I.14.1.8. Sunitinib (Sutent®)

Sunitinib is an oral small molecule multi targeted TKI approved by the FDA for treatment of renal cell cancer and imatinib resistant GIST (gastrointestinal stroma tumour). It inhibits signaling via PDGFR, kit, FLT3 and VEGFR2. A phase II trial of sunitinib 50mg orally daily for 4 weeks out of a six weeks treatment cycle in patients with NSCLC has been conducted. Out of 63 patients with previously treated, metastatic NSCLC 9.5% achieved partial response and 43% of patients had stable disease. Median survival was 23.9 weeks with a progression free survival of 11.3 weeks. Two patients with squamous cell histology had fatal pulmonary bleedings and one patient had a fatal central nervous system hemorrhage (16, 36).

I.14.2. Vascular disrupting drugs (VADs)

They are targeting pre-existing tumour capillaries leading to rapid cancer tissue ischemia and secondary tumour cell death in central tumour regions. The effect of VADs does not depend on vascularization compared to anti-angiogenic therapies. Small molecule VADs, leading to selective occlusion of tumour vessels because of phenotypic differences between tumour and host tissue endothelial cells must be distinguished from ligand directed VADs. Ligand directed VADs are toxin-containing and pro-coagulant agents coupled to peptides or antibodies that selectively bind to the endothelial tube. The latter are still in pre-clinical studies, therefore we focus on small molecule VADs (35).

I.14.2.1. 5,6 dimethylxanthenone-4-acetic acid (DMXAA)

It is a flavonoid VDA that has been demonstrated to augment anticancer effects of radiation, paclitaxel and cisplatin. The combination of DMXAA with carboplatin and paclitaxel is currently undergoing phase II evaluation for stage IIIb or IV NSCLC, previously untreated with chemotherapy. This combination could provide additional benefit compared to chemotherapy alone (35).

There had been fears that reduction of blood flow in tumours could have a negative influence on the delivery of chemotherapeutics and may reduce intratumoural oxygen levels essential for radiotherapy. But the above studies proved that combination of chemotherapy and angiogenesis inhibitors have additive or even synergistic anticancer effects. Possible explanations for this phenomenon are the normalization of tumour vasculature through the use of anti-angiogenic agents. Tumour vessels generally are characterized by leakiness, tortuosity and an increased interstitial pressure making cancer capillaries insufficient for providing adequate blood supply to the cancer cells. Blockade of VEGF signaling via bevacizumab leads to a normalization of vasculature and therefore improves the delivery of chemotherapeutic compounds. Another theory is that in presence of VEGF and VEGFR in cancer cells VEGF may serve as an autocrine growth factor. These autocrine loops enhance the malignant potential of NSCLC cells. Therefore combination of chemotherapy and angiogenesis inhibitors also has a direct cytotoxic effect, with VEGF blockers breaking down this autocrine loops. Last but not least anti-angiogenic agents prevent cancer cell repopulation during break periods of chemotherapy cycles (35).

The problems in the field of anti-angiogenic drugs are that clinical experiences are still limited. There are still problems in defining the optimal biologic dose of these agents and finding the best possible treatment combination strategies. Also toxicity profiles over long time periods are not available at the moment (35).

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

Table 1: Stage grouping for lung cancer

I.15. Tumour Hypoxia

Oxygen can diffuse 100 – 150µm from a blood vessel into surrounding tissues. Tumour cells located in more remote areas from a vessel are not able to survive. Due to the irregular blood vessels of tumours, the blood flow varies widely over the time, leading to states of acute and chronic hypoxia in tumour cells. In order to grow larger than 2mm in diameter or to metastasize, a network of new blood vessels must be formed to supply oxygen and nutrients. Exceptions are tumours that grow in the alveolar airspace and use the host's alveolar blood supply for growth and may therefore also not be sensitive for anti-angiogenic therapies. Hypoxia also affects cancer therapeutics. The concentrations of systemic chemotherapy behave similar to oxygen tensions and are lower at greater distances from a vessel. Hypoxic tumour cells are less metabolically active and divide more slowly than oxygen-rich cells, leading to relative resistance to cytotoxic chemotherapies. The hypoxia inducible transcription factors HIF1 alpha and HIF2 alpha become more stable in the setting of low oxygen tensions. Under hypoxia increased HIF activity activates transcription of genes, which help to respond to this stressor, initiating angiogenesis or anaerobic glycolysis for example. HIF activation has varying and contradictory effects on tumour cell survival. On one hand HIF activation leads to increased p53 apoptotic activity, on the other hand it increases VEGF secretion, interpreted as growth signal (36).

II. IMMUNOHISTOCHEMICAL STUDY: THE ROLE OF HYPOXIA INDUCIBLE FACTOR IN ANGIOGENESIS OF MALIGNANT LUNG TUMOURS

II.1. Abstract

Lung cancer is still the most common malignancy and leading cause of cancer death worldwide. The aim of this study was to identify the role of hypoxia inducible factor 1 alpha (HIF1 alpha) in angiogenesis of malignant lung tumours. We performed a retrospective immunohistochemistry study on tissue microarrays for adenocarcinomas, squamous cell carcinomas, large cell carcinomas, pleomorphic carcinomas, large cell neuroendocrine carcinomas and small cell lung carcinomas. The expression of eleven parameters (factors of hypoxia and angiogenesis) – HIF1 a, CAIX, TIE2, VEGFA, VEGFB, VEGFC, VEGFD, VEGFR2, VEGFR3, PDGFRa and PDGFRb – was evaluated by light microscopy. Product scores composed of intensity and percentage of stained cells were calculated for statistics. Statistic analyses provided boxplots, Wilcoxon's rank sum test comparing expression patterns between NSCLC subentities and SCLC, and Goeman's Global Test in order to compare NSCLC subtypes for preferences in activation of the above mentioned factors. Significant differences in activation of signaling pathways between all five NSCLC subtypes do exist. In adenocarcinomas and large cell neuroendocrine carcinomas the expression of VEGFC/VEGFD – VEGFR3 signaling was strongest. Large cell carcinomas showed highly activated levels of VEGFA, VEGFD, VEGFR3 and PDGFRa. Pleomorphic carcinomas were characterized by a high expression of all tested parameters except VEGFB and TIE2. Squamous cell carcinomas showed a preference for VEGFC/VEGFD – PDGFRb signaling in angiogenesis. SCLC followed no typical expression pattern of angiogenesis growth factors and receptors and seem to differ strongly from NSCLC signaling, again providing arguments for their different behavior in contrast to NSCLC. Generally, HIF1 alpha staining reactions were much lower than those for VEGFA/C/D and VEGF receptors expression, except in case of pleomorphic carcinomas, which showed nearly similar staining reactions for HIF1 alpha and the VEGF family. However, our statistics do no provide direct associations between HIF and VEGF signaling.

It seems that other proteins besides HIF may be involved in the up-regulation of angiogenesis as well and may play an even more important role.

II.2. Introduction

The incidence of lung cancer is increasing worldwide. Lung cancer is the primary cause of death among malignant tumours for both men and women. Once diagnosed, the 5-year-survival-rate of patients is not higher than 15% (1). In metastatic cases, prognosis remains poor and chemotherapy only minimally increases overall survival. Response rates to combined chemotherapy regimens are approximately 19%. There is an urgent need for novel therapies in the treatment of NSCLC. Even in surgically resectable cases, more than half of patients go on to develop metastatic disease within the next five years (16).

The above facts show that further research in the field of carcinogenesis is absolutely necessary. Intracellular molecular processes regulating tumour growth, proliferation, angiogenesis, metastasis or apoptosis have not been decoded exactly so far, but seem to be an interesting and new target for pharmaceutical strategies in the therapy of lung cancer.

The important role of tyrosine kinase receptors and their following signaling pathways in the development of lung cancer has been investigated in several phase II and III trials. Inhibitors of tyrosine kinases combined with standard chemotherapy have improved survival rates of patients in many clinical studies (1, 37-40). Examples for tyrosine kinase inhibitors are: Erlotinib (EGFR TKI), Sorafenib (VEGFR, PDGFR, c-kit TKI) and Sunitinib (VEGFR, PDGFR, KIT TKI). Especially the last two TKIs seem to be very promising drugs. Since they block several pathways simultaneously, there are fewer possibilities for tumour escape mechanisms left (1, 40).

Another interesting aspect is the behavior of tumours in hypoxic conditions. Hypoxia, basically, should lead to shrinking or even vanishing of tumours. But also in this case carcinomas have found a way to guarantee their survival, which runs over the hypoxia inducible factor, short HIF, a transcriptional factor inducing production of proteins, which regulate pro-survival processes in tumour cells like angiogenesis, vasodilatation, erythropoiesis, anaerobic metabolism and production of growth factors (21, 22, 24, 41).

Especially the clonal selection of cells with increased malignant potential seems to play an important role in tumour aggressiveness. High levels of hypoxia, therefore, lead to progression of tumours and are associated with poor prognosis, which has already been demonstrated for carcinomas of the cervix uteri (42). The problem of hypoxia is also the slower rate of tumour cell proliferation, which might protect them against conventional radiation and chemotherapy (42). Response to standard chemotherapy treatment under high hypoxic conditions is rather poor and survival rates cannot be increased. Besides the above-mentioned TKIs, also HIF could represent a novel tumour specific target for anticancer therapy in NSCLC. The combination of cytotoxic drugs like cisplatin and HIF1 alpha inhibition may have a synergistic anticancer effect, especially in metastatic or high stage disease (43).

The mechanisms of the activation of HIF, its downstream signaling cascades, its target genes and proteins and correlations between other molecular pathways have not been elucidated so far and are investigated in this study by immunohistochemistry on tissue microarrays of NSCLC subtypes and SCLC (see table 2). Due to the diverging results of other research groups (22, 23, 44-46), we especially look closely on the effects of HIF on angiogenesis in malignant lung tumours. Expression patterns of HIF1a, CAIX, VEGF, VEGFB, VEGFC, VEGFD, VEGFR2, VEGFR3, TIE2, PDGFRa and PDGFRb (see table 3) are investigated and then compared to existing reports. Furthermore we determined the 5-year-survival-rates for histological subtypes of lung cancer related to the primary tumour size.

Targets for the blockade of HIF1 alpha are microtubules and topoisomerase 1, however, their mechanisms of interaction with HIF1 alpha are still unclear. Pre-clinical studies are running on this matter. YC1, an inhibitor of platelet aggregation, suppresses HIF1 alpha expression in animal experiments and seems to take in a leading role in anti-tumour therapies. Concerning inhibition of CAIX as downstream factor of HIF, membrane bound aromatic sulfonamides inhibiting CA isoenzymes are tested in vitro for NSCLC in pre-clinical studies. Indisulam is one example, which is now in Phase I – II studies for solid tumours (41).

Cellular adaptation to hypoxia and altered glucose metabolism are fundamental to the basic biology and treatment of cancer. There are four lines of evidence for this hypothesis (47):

 Clonal expression of cancer cells depends on enhanced glucose transport and glycolysis (Warburg effect).

 Tumours cannot grow beyond several mm^3 without angiogenesis because of the limited diffusion of O2, glucose and nutrients.

 The degree of vascularization is inversely correlated with patient survival.

 The probability of invasion, metastasis and death are positively correlated with the degree of intratumoural hypoxia.

However, the architecture of microcirculation is defective and cells adjacent to neo vessels may often be hypoxic. Overall, tumour hypoxia is associated with resistance to chemotherapy, immunotherapy and radiotherapy (42, 47).

II.3. Materials and Methods

Immunohistochemistry was performed on four TMAs (AC, SQCC, LC/pleomorphic carcinomas and SCLC/LCNEC – see table 2), already existing from former IHC (immunohistochemistry) studies. Tissue cores for the TMAs were obtained from paraffin wax blocks of routinely processed and formalin fixed, surgically removed lung cancer samples. There are around 479 punches of tumour tissue, parenchyma and lymph node metastases coming from 50 to 75 patients on each TMA block. Each tumour is represented by 5 punches and one normal adjacent parenchyma punch on average. In order to demonstrate the presence of HIF1 alpha protein and CAIX as a downstream factor of HIF, new paraffin sections of the TMAs have been made.

HIF1 alpha mouse monoclonal IgG antibody, clone H 715 from Santa Cruz, dilution 1:1000, and CAIX polyclonal rabbit IgG antibody, clone H 120 from Santa Cruz, dilution 1:40, were used for protein detection and immunohistochemical staining reaction. Furthermore we used immunohistochemical data for angiogenesis factors (VEGFA, VEGFB, VEGFC, VEGFD) and their receptors (TIE2, VEGFR2, VEGFR3, PDGFRa and PDGFRb) from previous microarray studies (see table 3). Staining was then assessed semi-quantitatively by light microscopy, including the evaluation of the intensity of staining by a range from 0 – 3 (0= negative/no staining reaction, 1 = weak staining reaction, 2 = moderate staining reaction; 3 = intense staining reaction) and the percentage of positive cells for each core (compare also figures 4 and 5). Investigators were blinded to the distribution of cores from the same cases within the TMA. Staining of parenchyma cores (protein expression in alveolar, bronchial/-iolar epithelium, inflammatory and structural cells like fibroblasts) was not recorded. Macrophages were excluded from analysis due to their phagocytic capacity and problems of non-specific staining. The obtained values were fed into Microsoft Excel tables and the product of the semi-quantitative grading (0 – 3) and the percentage of cells affected in each punch was calculated for every single core. Finally, the arithmetic mean among cores belonging to the same case was determined. Afterwards, results were analyzed by statistics and expression patterns of HIF1 a, CAIX, TIE2, VEGFA, VEGFB, VEGFC, VEGFD, VEGFR2, VEGFR3, PDGFRa and PDGFRb were calculated for NSCLC and SCLC. Considering the histological subtypes (AC, LC, LCNEC, pleomorphic carcinoma, SCLC, SQCC), the minimum, first quartile, median, arithmetic mean, third quartile and the maximum concerning product score, intensity and percentage of stained tumour cells was calculated for each of the above mentioned eleven antibodies. The data are shown in box plots (compare figure 6 – 8). We decided to investigate the co-presence of hypoxic factors and factors of angiogenesis in lung cancer tissues in order to find out, whether HIF plays a role in initiation and acceleration of angiogenesis or not. (Statistic analysis provided by Dr. Franz Quehenberger, Institute for Statistics of the Medical University Graz).

Wilcoxon's rank sum test was approached to demonstrate significant differences of the individual expression of immunohistochemical parameters between NSCLC (AC, LC, SQCC, LCNEC and pleomorphic carcinomas) and SCLC. In order to achieve that, each histological subtype of NSCLC (AC, LC, SQCC, LCNEC and pleomorphic carcinomas) was calculated against SCLC concerning activation of hypoxic and angiogenic factors.

The null hypothesis that there are no differences in expression of signaling factors between NSCLC and SCLC was definitely disproved. Even Bonferroni adjusted p values [i.e. multiple testing by multiplying the p value by the number of tests (in our case p value multiplied by 12)] and determination of Q values [i.e. the probability that a statistically significant test with a p value below 0.05 is false positive (in our case we accepted 5% of false positive testing at maximum to be still statistically significant)] led to values below 0.05 which are regarded as statistically significant and cannot be explained by accident.

We used Goeman's Global Test (a global test for group of genes) (48) to compare histological subtypes concerning expression of immunohistochemical parameters. In order to do so all eleven antibodies together were tested between two groups, respectively: SCLC vs. SQCC product, SCLC vs. AC product , SCLC vs. LC product, SCLC vs. LCNEC product, AC vs. SQCC product, LC vs. SQCC product, LC vs. AC product, LCNEC vs. SQCC product, LCNEC vs. AC product , and LCNEC vs. LC product.

Histological Subtype	Number of Cases on the TMA
Adenocarcinoma	74
Large cell carcinoma	49
Large cell neuroendocrine carcinoma	24
Pleomorphic carcinoma	15
Small cell lung carcinoma	31
Squamous cell carcinoma	68
Total amount of lung carcinoma cases included in the IHC study:	261

Table 2 Lung carcinoma cases included in the study

Antibody	Clone	Company	Dilution	Pretreatment
Hypoxia inducible factor 1 alpha (HIF1 a)	H-715	Santa Cruz	1:1000	CC1
Carbonic anhydrase IX (CAIX)	H-120	Santa Cruz	1:40	CC1
Vascular endothelial growth factor (VEGF)	A-20	Santa Cruz	1:500	MW LSAB
Vascular endothelial growth factor B (VEGFB)	58013	R&D	1:300	MW 6.0
Vascular endothelial growth factor C (VEGFC)	N.A.	R&D	1:200	MW Tris
Vascular endothelial growth factor D (VEGFD)	78923	R&D	1:40	MW 6.0
Vascular endothelial growth factor receptor 2 (VEGFR2)	SC-6251	Santa Cruz	1:50	CC1
Vascular endothelial growth factor receptor 3 (VEGFR3)	SC 321	Santa Cruz	1:200	CC1
TIE2	H-176	Santa Cruz	1:50	CC1
Platelet derived growth factor receptor a (PDGFRa)	N.A.	Neomarker	1:50	KT 6.0
Platelet derived growth factor receptor b (PDGFRb)	N.A.	Neomarker	1:100	MW 9.0

Table 3: Antibodies used for the study; Abbreviations: N.A. = not available; CC1=cell conditioning buffer 1 prediluted from Ventana; MW LSAB: microwave peroxidase blocking solution from Dako; MW 6.0 = microwave natriumcitrate buffer pH 6.0; MW Tris: microwave Tris-HCl-buffer + 5% Urea, pH 9.0; KT 6.0= heat steamer pH 6.0 ; MW 9.0 = microwave target retrieval solution pH 9.0 from Dako

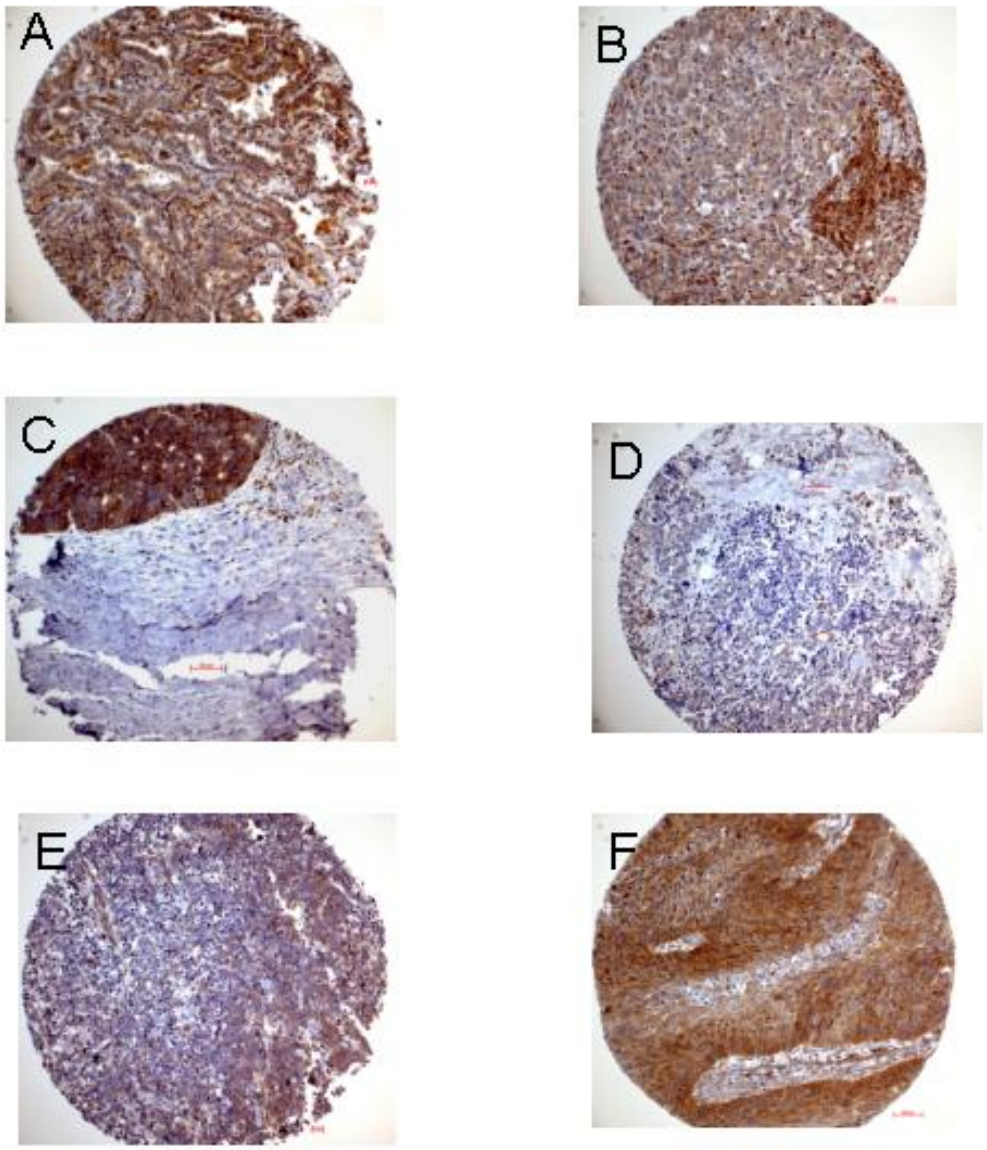
II.4. Histological Results of HIF1 a/CAIX expression in lung carcinoma tissue

Alveolar macrophages, plasma cells, bronchus epithelial and endothelial cells in normal lung parenchyma were stained highly positive with the HIF1 alpha antibody used. Tumour infiltrating macrophages and stromal cells also showed high positive staining in nuclei and cytoplasm. Their immunohistochemical reaction was in most cases stronger than that of the tumour cells. Tumour cells in a high percentage expressed HIF1 alpha protein in the cytoplasm and the intensity of staining was rather variable among all histological subtypes and cases (see figure 4). Nuclear staining was found only in a minority of cases and among them only 1 – 3% of tumour cells expressed HIF1 alpha protein in their nuclei. Nuclear staining was more common among adenocarcinomas, large cell and pleomorphic carcinomas, and small cell carcinomas. Squamous cell carcinomas showed almost no nuclear reaction.

There have been diverging arguments, whether it would be valid to define cytoplasmic IHC staining of HIF1a as positive, as only nuclear HIF1 alpha is the active form. Lee et al (44) strictly counted only nuclear staining of tumour cells as positive, however 1% of nucleic staining was sufficient to be evaluated as positive. Swinson et al (22) also chose to take only nuclear staining into their statistic analysis. Again, > 5% of nuclear tumour cell staining was already determined as positive and only one fifth of the cases positive for HIF1 alpha reached the high cut-point of > 60% nuclear staining. Hirami et al (45) considered both nuclear and cytoplasmic staining for HIF1 alpha as positive. Giatromanolaki et al (23) explained that although nuclear HIF is assumed as active form, its synthesis and degradation takes place in the cytoplasm and therefore also reflects the hypoxic answer and up-regulation of this pathway. Their analysis based on pure nuclear HIF1 alpha expression showed very marginal or no statistical association with other molecular factors of prognosis like T-stage, N-stage or MVD. They think that some kind of HIF1 alpha redistribution could take place, while gaining the tissue samples and formalin fixation and therefore cytoplasmic staining of HIF would better reflect the up-regulation of the pathway in paraffin-embedded material (23).

We also think that surgical resection might have an influence on HIF1 alpha distribution. When arteries are clipped off, the blood flow is stopped from one second to another leading to acute hypoxic conditions.

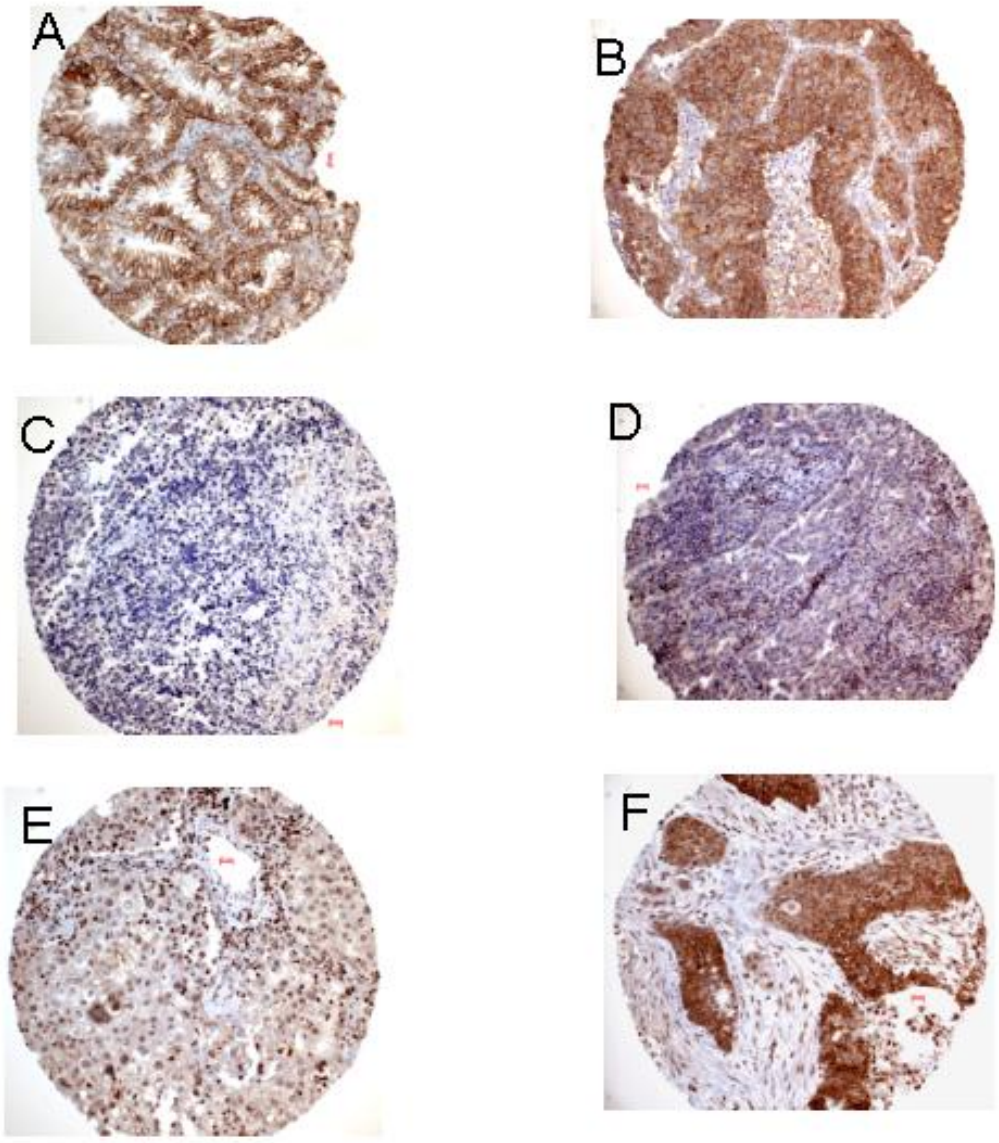
HIF1 alpha will then be highly synthesized in the cytoplasm, enters the nucleus and promotes transcription of genes responsible for anaerobic metabolism or neo-angiogenesis. All these steps take place within seconds and HIF1 alpha may soon be degraded afterwards. There could be also fluctuations in blood flow during operations leading to effects like activation and degradation of HIF1 alpha. Furthermore fixation with formalin could also have an influence on HIF1 alpha protein distribution. Strong cytoplasmic HIF expression seems to be more common in tumours (47). If this better reflects the HIF up-regulated pathway in paraffin-embedded material is still not solved.



A-F: Staining reactions performed by HIF 1 alpha mouse monoclonal IgG antibody, clone H-715 from Santa Cruz. A: AC with typical nuclear and cytoplasmic staining, intenstiy grade 3, 40% of cells stained. B: LC with mainly cytoplasmic staining, intensity grade 2, 90 % of cells stained and strong stromal staining reaction of intensity grade 3. C:LCNEC with strong nuclear and cytoplasmic staining, intensity grade 3, 90% of tumour cells stained. D:SCLC with weak cytoplasmic staining for HIF 1 a, intensity grade 1, 60% of tumour cells stained. E: SCLC with mainly cytoplasmic staining, intensity grade 2, 80% of tumour cells stained. F: SQCC with cytoplasmic staining, intensity grade 3, 60 % of tumour cells stained.

Figure 4: Punches of NSCLC and SCLC with different staining reactions performed by HIF1 alpha mouse monoclonal IgG antibody.

Concerning the staining of CAIX in tumour tissues, immunohistochemical reaction was typically membranous and cytoplasmic in NSCLC and SCLC cases which underlines the membranous location of this enzyme (see figure 5). In some cases also strong nuclear staining of tumour cells was found. There was no or only rare staining for CAIX in bronchial epithelial and endothelial cells in normal lung tissue.



A-F: Staining reactions performed by CA IX polyclonal rabbit IgG antibody, clone H120 from Santa Cruz. A: AC with typical membranous and cytoplasmic staining, intensity grade 3, 90% of cells stained. B:LCNEC with typical membranous and cytoplasmic staining, intensity grade 3, 70% of cells stained. C: SCLC with negative staining for CAIX; D: LC with cytoplasmic staining; intensity grade 2, 90 % of tumour cells stained. E+F: Two cases of SQCC. E: Weak staining pattern with intensity grade 1, 90% of cells stained. F: Strong staining pattern , intensity grade 3, 60% of cells stained.

Figure 5: Punches of NSCLC and SCLC with different staining reactions performed by CAIX polyclonal rabbit IgG antibody.

II.5. Statistical Results

II.5.1. Results according to immunohistochemical parameters (based on product score; compare also boxplot figure 6)

VEGFR3 expression was intense in pleomorphic carcinomas, large cell carcinomas and adenocarcinomas and median of product scores lies close together around 200. Lower values are found among SCLC and LCNEC (median of product scores around 50). SQCC showed the lowest rate of VEGFR3 expression.

VEGFR2 expression is generally much lower in all kind of lung carcinomas compared to VEGFR3 expression. It was negative in LCNEC and plays only a minor role in SCLC and SQCC. Somewhat higher expression patterns were seen in AC, LC and pleomorphic carcinomas (median of product scores between 20 and 50).

VEGFD was highly up-regulated in all histological subtypes with a median of product scores lying in between 180 – 200.

VEGFC shows a similar expression pattern to VEGFD, the median of the product scores was a bit higher in pleomorphic carcinomas, AC and LCNEC, amounting to 150 and a little bit lower in SCLC, SQCC and LC, running to 100.

VEGFB expression was negative in almost every tumour; there were only one or two positive cases among AC, LC and pleomorphic carcinomas respectively, which is statistically insignificant.

VEGFA was expressed differently among lung carcinomas. Highest expression was found in pleomorphic carcinomas and LC with a median of product scores around 125, followed by SQCC with a median of 80 and lowest expression in SCLC, LCNEC and AC with a median around 50.

 TIE2 seems to play a minor role in signaling pathways of lung carcinoma with only a few positive cases among SQCC, SCLC and LCNEC. Some high mavericks were found among AC, LC and pleomorphic carcinomas with rather high values. However, the role of TIE2 receptor and its angiopoietin ligands in tumour growth and angiogenesis seems to be statistically insignificant in the case of lung cancer.

 PDGFRb expression patterns also varied among histological subtypes. It seems to be a typical signaling pathway in pleomorphic carcinomas, SQCC and LC, with a median of the product scores of 160 for pleomorphic carcinomas, and of 100 for SQCC and LC. PDGFRb expression seems to be less important in AC, LCNEC and SCLC, with a median between 10 and 25, respectively.

 PDGFRa was up-regulated the most in pleomorphic carcinomas with a median of the product scores of 175, followed by LC with a median of 120. A lower median was found among AC and SQCC (75), LCNEC (50) and SCLC (10).

 HIF a was similarly intense expressed in all types of lung carcinomas. The median of the product scores for AC, LC, LCNEC and SQCC closely grouped together at 75. Expression was much higher in pleomorphic carcinomas (median of 150) and rather low in SCLC (median of 40).

 CAIX expression goes along with results of HIF1a expression, however medians were lower than those of HIF1 a, being around 50 for all subtypes except SCLC which had a median of the product scores of only 5.

II.5.2. Results according to histological subtypes (based on product scores, compare also boxplot figure 6)

In adenocarcinomas the combined VEGFC/VEGFD and VEGFR3 ligand/receptor activation was prominent and might promote the activation of the angiogenesis pathway leading to lymphangiogenesis and lymphatic metastases.

Large cell carcinomas were characterized by high levels of PDGFRa, VEGFA, VEGFD and VEGFR3 expression. Angiogenesis seems to run via PDGF and VEGF receptors.

Concerning LCNEC also a strong expression of VEGFC, VEGFD and VEGFR3 could be found.

Pleomorphic carcinomas are the histological subtype with the highest up-regulation of nearly all the parameters tested. PDGFRa, PDGFRb, VEGF, VEGFC, VEGFD, VEGFR3 and HIF1a were highly expressed. Only VEGFB and TIE2 expression was rather low. This type of carcinoma may choose many different pathways for neoangiogenesis.

Among SCLC no typical expression pattern of hypoxic and angiogenic factors could be demonstrated. Some tumours expressed them at very high levels others at very low ones or even none of them at all.

In SQCC up-regulation of PDGFRb, VEGFC and VEGFD was commonly found. VEGFR3 however, was only expressed in one of the cases. Therefore angiogenesis in SQCC might signal via PDGF receptors.

II.5.3. Results according to histological subtypes (based on intensities, compare also boxplot figure 7)

In adenocarcinomas highest intensities could be achieved in VEGFD expression (intensity grade 2 on average). Large cell carcinomas showed the strongest staining reactions in VEGFD and VEGFR3 protein detection (intensity grade 2 on average).

In LCNEC highest values of intensity were achieved by VEGFC and VEGFD staining (grade 1.8 and 2 on average). Pleomorphic carcinomas reached high intensities in PDGFRa (grade 2), VEGFD (grade 2.1), and VEGFR3 (grade 2) expression, respectively. SCLC achieved highest intensity in VEGFD expression (grade 1.8), and intensity of SQCC staining was highest for VEGFD (grade 2).

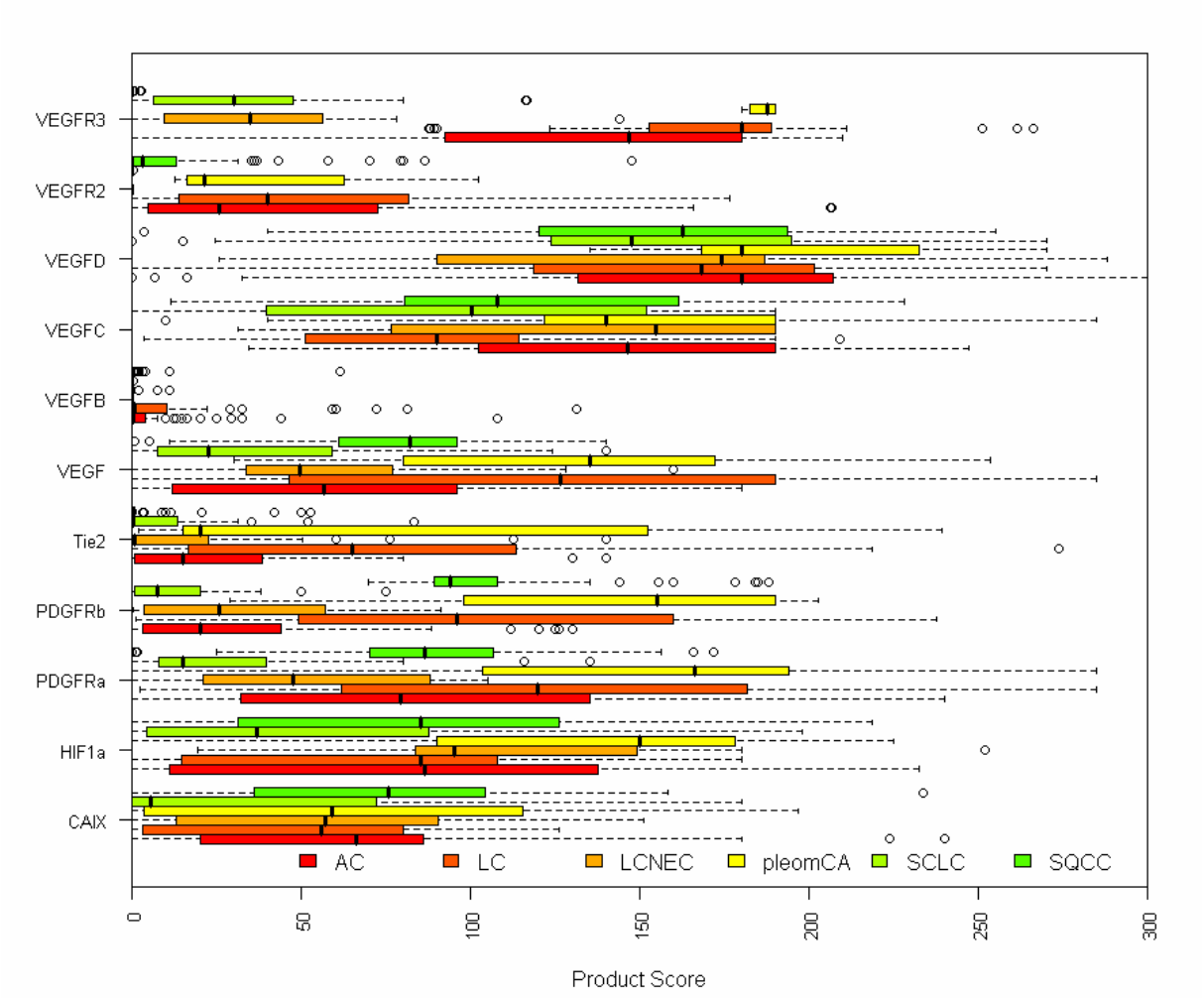


Figure 6: Boxplot containing product scores of immunohistochemical parameters in NSCLC and SCLC (absolute values)

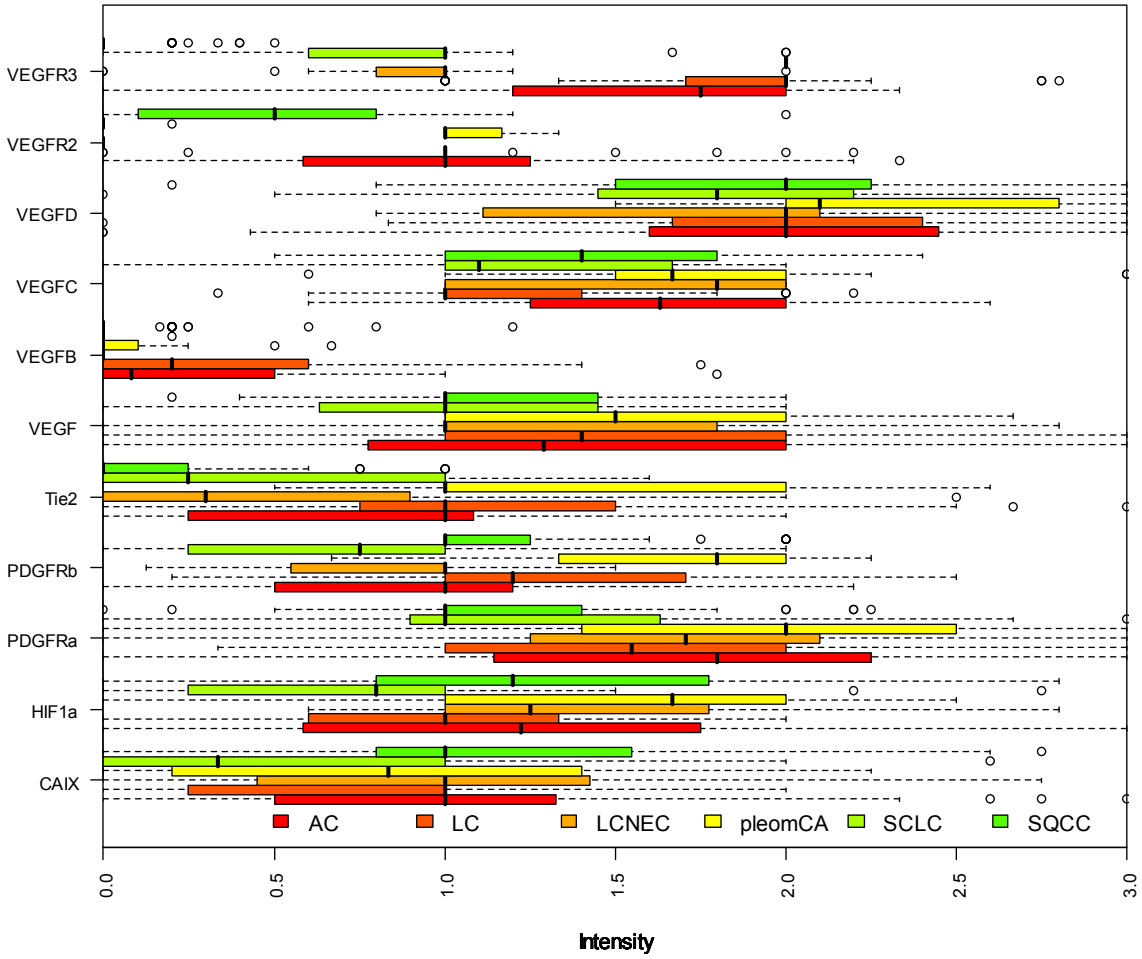


Figure 7: Boxplot containing intensities of immunohistochemical parameters for NSCLC and SCLC.

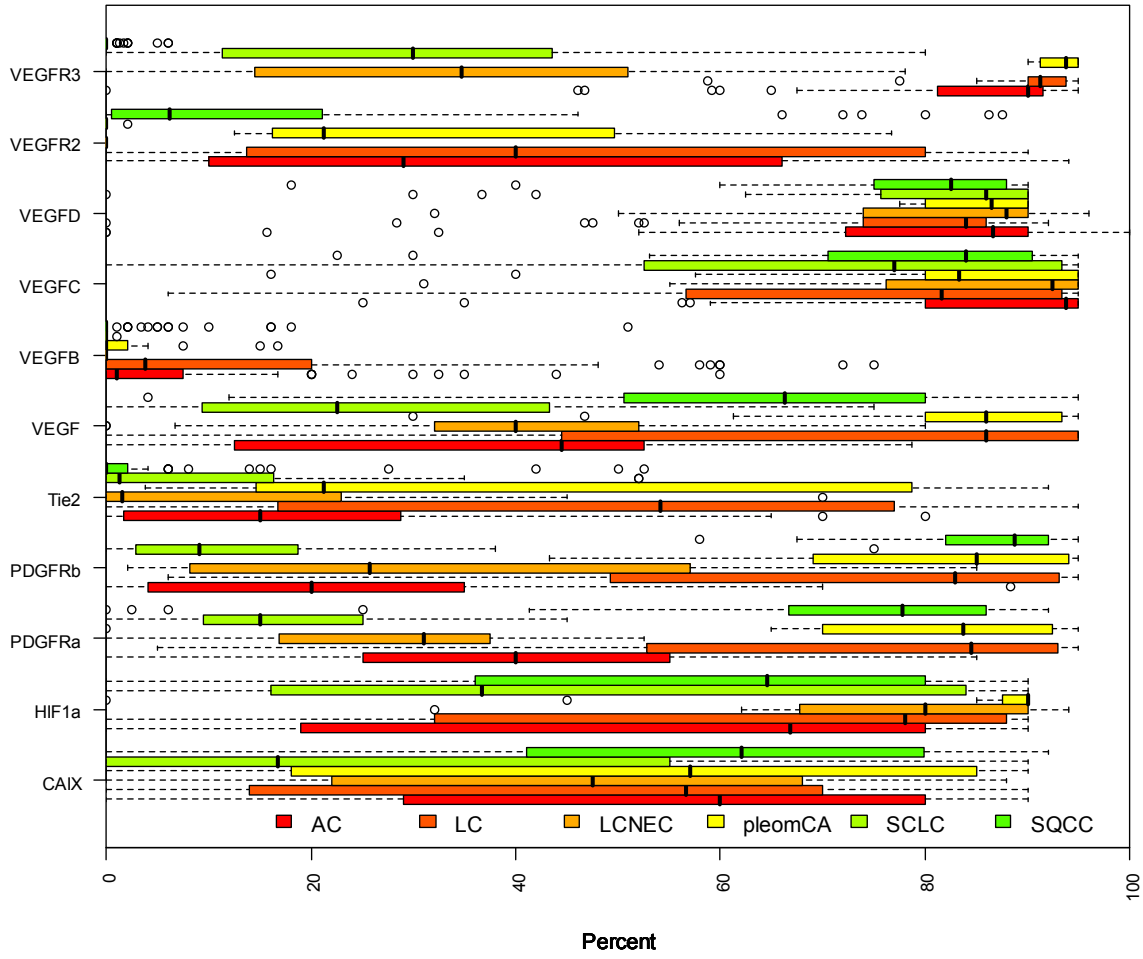


Figure 8 : Boxplot containing percent of stained tumour cells for immunohistochemical parameters in NSCLC and SCLC.

II.5.4. Results of Wilcoxon Test

Generally, there are many significant differences in up-regulation of signaling pathways between all kind of NSCLC and SCLC. Most significant differences of expression patterns could be found between:

- LC vs. SCLC [significant differences in VEGFR3 (p=4.5e-17), VEGFR2 (p=1.0e-14), PDGFRa (1.0e-7), PDGFRb (5.6e-11), VEGFA (2.1e-5), VEGFB (1.2e-5) and TIE2 (6.2e-6) expression, 7 of 11 parameters tested, regarding Bonferroni corrected p values]

 Pleomorphic carcinomas vs. SCLC [significant differences in VEGFR3 (p= 0.00042), VEGFR2 (p=0.00021), PDGFRb (p=3.6e-9), PDGFRa (p=0.000022), VEGFA (p=0.000081), TIE2 (p=0.00092) and HIF1a (p=0.0026); 7 of 11 parameters statistically significant regarding Bonferroni corrected p values]

followed by:

 AC vs. SCLC [significant differences in VEGFR3 (p=2.7e-12), VEGFR2 (p=3.5e-12), PDGFRa (0.000015), VEGFB (p=0.000067) and VEGFC (p=0.011); 5 of 11 parameters statistically significant regarding Bonferroni corrected p values]

and

 SQCC vs. SCLC [significant differences in VEGFR3 (p=4.9e-15), VEGFR2 (p= 6.0e-9), PDGFRb (p=2.2e-14), PDGFRa (p=2.0e-8) VEGFA (p=0.000048), CAIX (p=0.0036); 6 of 11 parameters statistically significant regarding Bonferroni corrected p values]

Only little statistically significant differences in expression of hypoxia and angiogenesis growth factors could be found between LCNEC and SCLC which might be traced back to histological similarities concerning neuroendocrine differentiation (4 of 11 parameters statistically significant regarding Bonferroni corrected p values).

Statistical non-significance between NSCLC and SCLC was noted for VEGFB, HIF1 a, CAIX, TIE2, VEGFC and VEGFD expression.

To sum up we can say that SCLC is a special entity of lung tumours, running different signaling pathways than NSCLC. Pharmacies for NSCLC treatment, like tyrosine kinase inhibitors affecting VEGFR, PDGFR pathways, might be not effective in SCLC.

II.5.5. Results of Goeman's Global Test

When comparing AC with SQCC it became clear that angiogenesis in the case of AC especially runs via high up-regulation of VEGFR3, VEGFR2 and TIE2, whereas SQCC induce new formation of blood vessels especially via strong expression of PDGFRb.
Also among large cell carcinomas the pathway following TIE2 – VEGFB – VEGFR2 seems to play a major role compared to SQCC signaling.

Overall, AC follows VEGFR signaling pathways for angiogenesis in the majority of cases, whereas SQCC mainly signal via PDGFR, and LC seems to have activated both VEGF and PDGF receptors to induce angiogenesis.

II.6. Survival Analysis

II.6.1. Survival Analysis Methods

For survival analysis we used Kaplan Meier survival curves (see figures 9 - 12). The Kaplan-Meier method is helpful for the determination of the probability to survive a certain time period from the date of diagnosis of a disease. The idea behind this method is that the event – in our case the death of a patient suffering from lung cancer included in the study – defines the time period of surveillance. There is no constant time period. A new period of surveillance starts with the death of a patient. Then for each interval the limited probability, that a patient survives it, gets calculated, provided that he/she is still alive at the beginning of the new time period. The total probability to survive a certain time can be described as the product of the limited probabilities for each time period. Kaplan Meier curves especially serve the purpose to read out specific survival rates like for example the 5-year-survival-rate. [www.rbsd.de/PDF/DMW/DMW-2007-S1-15.pdf]

In our study we determined and compared the 5-year-survival-rates (see table 8) for the different histological subtypes of lung cancer (AC, LC, pleomorphic CAs and SQCC – case numbers shown in table 4) related to the primary tumour size and spreading (pT 1 – 4 – explanations see table 5). Our surgical tissue samples mainly include pT1 and pT2 tumours as primary curative resection is only carried out in these cases. We included only a few pT3 and pT4 samples, which are rarely resected, but receive first line chemotherapy.

II.6.2. Results of survival analysis

T	Primary tumour
TX	Primary tumour cannot be assessed, or tumour proven by the presence of malignant cells in sputum or bronchial washings but not visualized by imaging or bronchoscopy.
T0	No evidence of primary tumour
Tis	Carcinoma in situ
T1	Tumour 3cm or less in greatest dimension; surrounded by lung or visceral pleura, without bronchoscopic evidence of invasion more proximal than the lobar bronchus, i.e. not in the main bronchus.
T2	Tumour with any of the following features of size or extent: More than 3cm in greatest dimension Involves main bronchus, 2cm or more distal to the carina Invades visceral pleura Associated with atelectasis or obstructive pneumonitis that extends to the hilar region but does not involve the entire lung
T3	Tumour of any size that directly invades any of the following: Chest wall (including superior sulcus tumours), diaphragm, mediastinal pleura, parietal pericardium; or tumour in the main bronchus less than 2cm distal to the carina but without involvement of the carina; or associated atelectasis or obstructive pneumonitis of the entire lung.
T4	Tumour of any size that invades any of the following: Mediastinum, heart, great vessels, trachea, oesophagus, vertebral body, carina; separate tumour nodules in the same lobe; tumour with malignant pleural effusion.

Table 4: Classification of primary tumour size and spreading in the lung (pT) according to the TNM classification of the UICC (International Union Against Cancer – www.uicc.org) (2).

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension
N2	Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)
N3	Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s)

Table 5: N-Regional Lymph Nodes according to the TNM classification of the lung of the UICC (2).

MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis, includes separate tumour nodule(s) in a different lobe (ipsilateral or contralateral)

Table 6: M – Distant metastasis according to the TNM classification of the lung of the UICC (2).

Primary Tumour	AC	SQCC	Pleom. CA	LC	Total number of cases included in survival analysis:
pT1	(n) 43	(n) 22	(n) 3	(n) 16	
pT2	(n) 27	(n) 37	(n) 11	(n) 31	
pT3	(n) 3	(n) 4	(n) 0	(n) 1	
pT4	(n) 0	(n) 1	(n) 0	(n) 1	
Total:	(n) 73	(n) 64	(n) 14	(n) 49	(n) 200

Table 7: Case numbers related to histological subtype and primary tumour size and spreading (pT) included in survival analysis

T-Size (pT)	AC	SQCC	LC	Pleomorphic carcinoma
pT1	55%	70%	65%	100%
pT2	40%	45%	58%	20%
pT3	3 patients included in the study with survival rates ranging from 2.5 years at maximum to only a few weeks at minimum.	75%	Only one patient included in the study, who survived one year from date of diagnosis.	No case included in the study.
pT4	No case included in the study.	Only one patient included in the study, who died within a year from date of diagnosis.	Only one patient included in the study, who died after 3.5 years from date of diagnosis.	No case included in the study.

Table 8: Results of survival analysis; 5-year-survival-rate for histological subtypes of NSCLC related to the primary tumour size and spreading.

In AC and LC decreasing 5-year-survival-rates correlated well with increase of tumour size. Interestingly, among SQCC pT3 tumour patients showed the highest 5-year-survival-rate with 75% as against patients with pT1 SQCC had a 5-year-survival-rate of 70%. Out of 22 pT1 tumours in SQCC, nine showed lymph node involvement (40%), whereas out of four pT3 tumours in SQCC, only one had positive lymph nodes (25%), which might be a possible explanation for longer survival rates in pT3 tumours. In addition we had to deal with a very small number of pT3 tumours, which might also serve to explain this divergence. Therefore a clear hypothesis about primary tumour-related survival rates in SQCC cannot be made. The 5-year-survival-rate for pleomorphic carcinomas showed a significant fall from 100% in pT1 tumours to 20% in pT2 tumours.

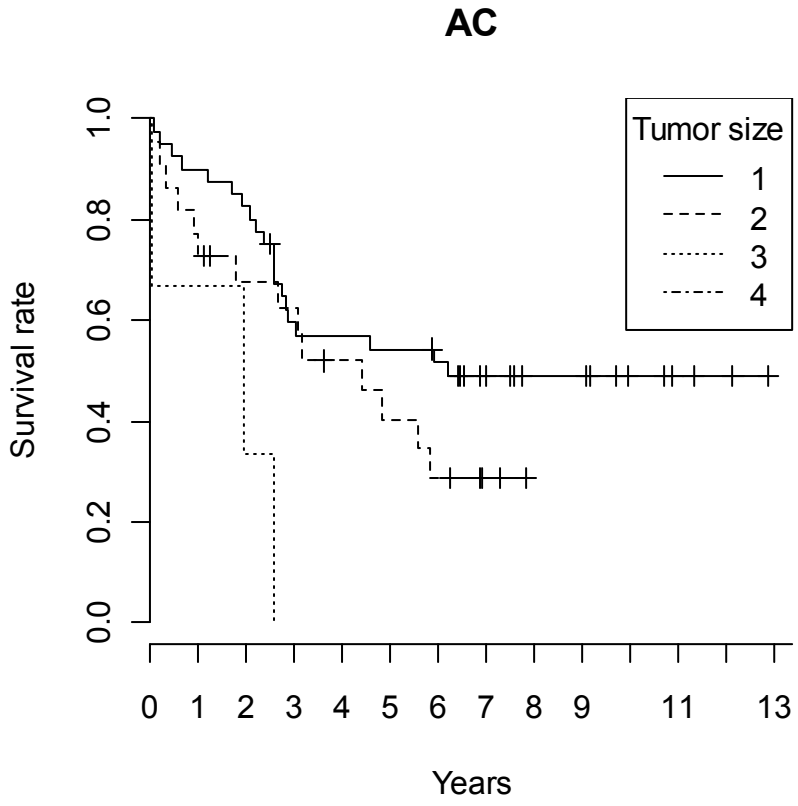


Figure 9: Kaplan Meier Curve for AC comparing pT1 - 4 cases.

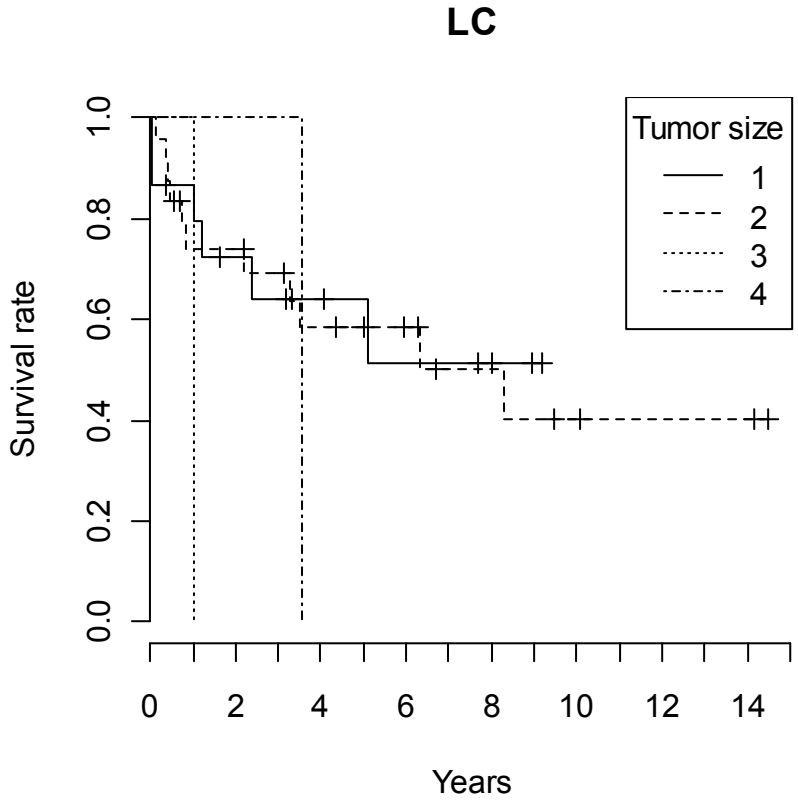


Figure 10: Kaplan Meier Curve for LC comparing pT1 – 4 cases.

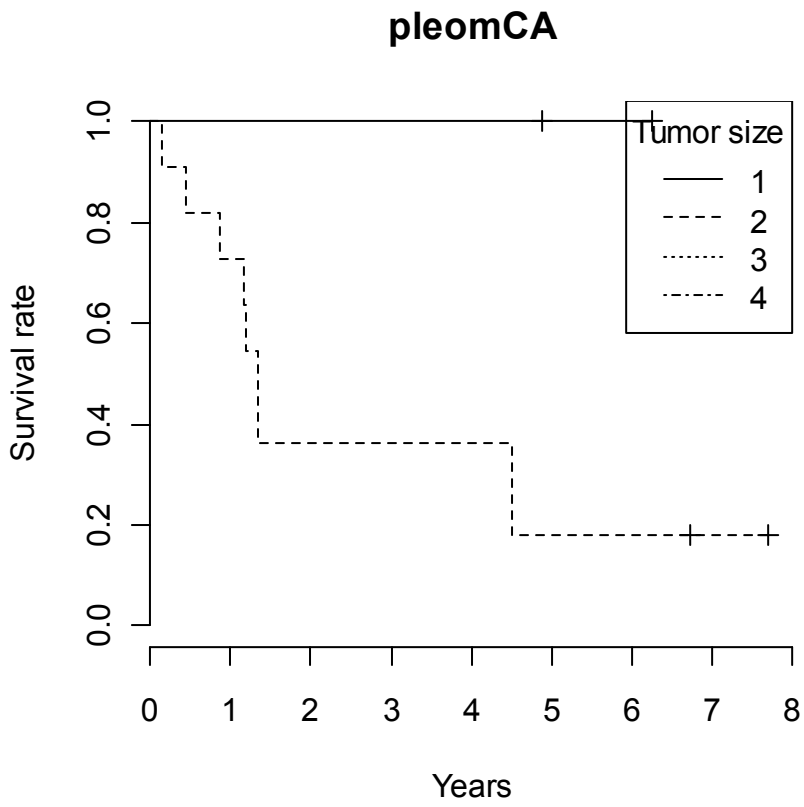


Figure 11: Kaplan Meier Curve for pleomorphic carcinomas comparing pT1 – 4 cases.

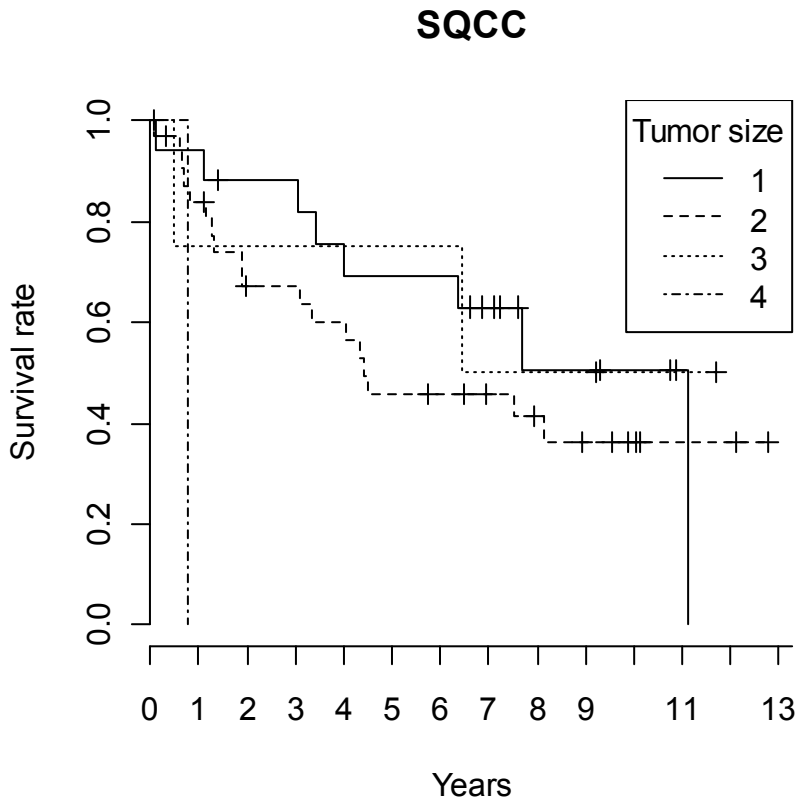


Figure 12: Kaplan Meier Curve for SQCC comparing pT1 – 4 cases.

II.7. Discussion

In our study differences between the histological subentities of lung cancer concerning activation of signaling pathways do exist and especially SCLC do not follow typical growth factor expression patterns compared to other subtypes. Adenocarcinomas and LCNEC expressed VEGFC, VEGFD and VEGFR3 highest among all the parameters tested and therefore pharmaceutical blocking of this angiogenesis pathway with tyrosine kinase inhibitors like Sorafenib or Sunitinib might make sense. Large cell carcinomas were characterized by high levels of PDGFRa, VEGF, VEGFD and VEGFR3 expression. In this case also intervention with tyrosine kinase inhibitors like Sorafenib or Sunitinib (both blocking VEGFR and PDGFR signaling) but also the use of Bevacizumab (Avastin®), a humanized monoclonal antibody against soluble VEGF which was recently approved for treatment of non-operable NSCLC (36) seems to be a possibility in cancer treatment among LC. Pleomorphic carcinomas had activated nearly all of the tested molecular signaling proteins, even HIF1 a was expressed strongly. Only TIE2 and VEGFB expression was rather insignificant. Therefore we would recommend the use of multi-targeted tyrosine kinase inhibitors against PDGFR, VEGFR, but also bevacizumab for the treatment of pleomorphic carcinomas. Even blockade of HIF1 alpha could be considered, however agents like microtubule-targeted agents or topoisomerase 1 inhibitors that downregulate HIF1 alpha are still in preclinical studies and their mechanisms of HIF1 alpha inhibition are currently unknown. Also YC-1, a platelet aggregation inhibitor now tested in animal models, has shown to have suppressive effects on HIF1 alpha activity. It has been postulated to become a leading compound in the development of a new class of anti-tumour agents (41).

Concerning SQCC the activation of the VEGFC/VEGFD-PDGFRb pathway was very common, VEGFR3 however was only expressed in one case, and therefore biological agents interfering with PDGFRb would be a potential therapy. SCLC had a rather varying pattern of growth factor and receptor expression, some showed very high expression of hypoxia and angiogenesis parameters others none, therefore only a certain group of SCLC patients could profit from monoclonal antibodies or tyrosine kinase inhibitors disturbing angiogenesis pathways.

However, statistic analysis of our study does not deliver a hypothesis concerning direct associations of HIF1 a and the investigated angiogenesis growth factors and receptors, as correlations between single factors (like HIF1 a and VEGFC/D) had not been calculated. We only demonstrated the expression of the above mentioned signaling proteins but did not correlate them among one another. Also the statement that HIF1 alpha is responsible for the activation and up-regulation of VEGF cannot be made with our data. We can only show that HIF1 a expression is decreased compared to VEGFA, VEGFC, VEGFD and VEGFR3 expression in AC, LC and pleomorphic carcinomas. SCLC, SQCC, and LCNEC however showed a lower expression of VEGFR3 compared to HIF1 a. We are sure that also other oncogenes such as activated EGFR, mutant ras and erbB-2/Her2 (35) may lead to an up-regulation of the VEGF pathway aside from tumour hypoxia and HIF1 alpha activity.

Immunohistochemical studies showing a direct relation between HIF1 alpha and the VEGF pathway have been performed. Giatromanolaki et al. (23) investigated the relation of hypoxia inducible factor 1 alpha and 2 alpha in operable non-small cell lung cancer to angiogenic/molecular profile of tumours and survival. They reported a very strong association of the expression of proteins (HIF1 alpha and HIF 2 alpha) with the angiogenic factors VEGF, PDGF and bFGF. Blockade of the HIF transcriptional pathway was assumed to be a potential target for cancer prevention. Furthermore, following inhibition of the HIF pathway there is no up-regulation of multiple angiogenic factors and over-expression of angiogenic receptors in the downstream cascade (23). Lee et al. (44) also examined the direct association between HIF1 alpha and VEGF with immunohistochemistry on paraffin-embedded tissue blocks, however they did not find any statistically significant relation. They only reported about significant differences concerning histological subtypes of NSCLC. SQCC expressed HIF1 alpha much more frequent than AC (44). In our study SQCC and AC expressed HIF1 alpha almost similarly and we can therefore not support the trend of higher HIF1 alpha expression in SQCC.

Lee et al. (44) came to the conclusion that hypoxia may trigger VEGF expression through HIF1 alpha stabilization but the process of angiogenesis in pulmonary tumours is also subject to other modulators such as PDGF, BCL2, c-erb B2 and MUC1 glycoprotein (44), which goes along with a statement of Giatromanolaki et al. (23) who also declare that hypoxia triggers the expression of a cascade of angiogenic factors through HIF stabilization, the process of angiogenesis however depends on a series of other modulators (23).

Giatromanolaki et al. (23) also mentioned that HIF1 alpha activation is expected to occur at a very early step of tumour progression which includes in situ carcinomas (23) or even metaplasia. Concerning early HIF activation in the case of lung tissue Polosukhin et. al (49) performed a very interesting study about the association of progressive structural changes in the bronchial mucosa. They examined the expression of HIF1 alpha and CAIX, the so-called hypoxia markers in lung resections of patients with COPD (chronic obstructive pulmonary disease). During the chronic progression of COPD different airway remodeling processes take place. They include hypertrophy and hyperplasia of smooth muscles, thickening and fibrosis of the subepithelial RBM (reticular basement membrane), enlargement of submucosal bronchial glands and structural alterations of the bronchus epithelium. Through thickening of the reticular basement membrane and the loss of subepithelian microvasculature less nutrients and oxygen can be transported to the epithelium, and as a result of this lack, normal epithelium transforms into squamous metaplasia. Therefore, they investigated, whether HIF1 alpha and CAIX as potential marker for hypoxia are present in these tissues and if they could be responsible for the different ways of remodeling. They found that the number of HIF1 alpha expressing cells is higher in areas of epithelial restructuring, increased RBM thickness and reduced capillary density in the submucosa. In patients with COPD the normal bronchial epithelium changes into pseudostratified, stratified immature metaplasia, squamous metaplasia, and dysplasia. HIF1 alpha expression was highly positive in all pathologic forms except pseudostratified/normal epithelium. The reaction in epithelial cells was cytoplasmic and nuclear. In altered/pseudostratified and stratified epithelium, HIF1 alpha was dominantly expressed in intermediate and surface cells. In squamous metaplasia, HIF1 alpha positive cells were especially found in the germinal zone (49).

CAIX was only expressed in squamous metaplasia and dysplasia. In squamous metaplasia, CAIX is expressed in the membranes of most basal epithelial cells. In dysplasia, CAIX was positive in membranes, nuclei and cytoplasm. Increased HIF1 alpha/CAIX expression in the bronchus epithelium could therefore be a sign for activation of hypoxic signaling pathways. Generally, HIF1 alpha was expressed more often than CAIX which might be due to the degree of hypoxia. The lack of oxygen for HIF1 alpha induction seems to be much lower than for CAIX induction. Squamous metaplasia and dysplasia seem to be those forms with the highest lack of oxygen, reflecting co-localization of HIF1 alpha and CAIX. In dysplasia, the thickness of the RBM decreases because of the digestion via MMP and the sub epithelial microcapillary density increases via induction of VEGF. However, the structure of these capillaries is abnormal and does not lead to sufficient blood supply. The results suggest that hypoxic signaling is a potential target to prevent progression of epithelial structural abnormalities, neoangiogenesis and, ultimately, the development of squamous cell lung cancer in high-risk individuals with COPD (49).

Also in our study CAIX expression played a minor role compared to HIF1 alpha expression which leads to the question, whether HIF1 alpha activation is really predominately a cause of hypoxia or if there might be other modulators of expression aside from lack of oxygen. Nature Reviews Cancer (50) published an interesting article about cycling hypoxia and free radicals regulating angiogenesis and radiotherapy response. It is known that tumour vessels have an abnormal structure which leads to spatial and temporal inadequacy in delivery of oxygen and other nutrients. When HIF is activated, it has two possibilities to act – it can switch either to angiogenesis or to anaerobic metabolism. The activity of the HIF is controlled by micro-environmental factors involving hypoxia, oxidative and nitrosative stress. It is regulated by many factors aside from hypoxia, including oncogenes, growth factors and free-radicals such as super oxide anion, hydrogen peroxide and nitritic oxide. The role of reactive oxygen species in the regulation of HIF1 alpha is at least as important as hypoxia per se and is far more complex than hypoxia itself.

There are seven reasons for hypoxia and deficiencies in oxygen transport of tumours:

- sparse arteriolar supply
- inefficient orientation of microvessels
- low vascular density
- variations in microvessel red blood cell flux
- limited arteriolar supply leading to pathologically low vascular pO_2 in regions distant from the arteriolar source (longitudinal oxygen gradient)
- hypoxic red blood cells are stiff and increase blood viscosity, contributing to a sluggish flow
- large diameter shunts divert blood away from the tumour

It is assumed that tumour hypoxia is unstable; there is cycling with clear patterns of periodicity. This cycling hypoxia leads to higher up-regulation of HIF1 alpha activity than chronic hypoxia and also increases free radicals (hypoxia-reoxygenation-injury). The extent and severity from hypoxia alters from day to day influencing the oxygen delivery to tumour micro regions. Concerning the relation between hypoxia – HIF and angiogenesis there are at present two models to be discussed (50):

The angiogenesis initiation model

It starts with Angiopoietin 2 over-expression without expression of VEGF, which causes a vascular regression. The regression is followed by hypoxia, which is then activating HIF up-regulation and VEGF expression downstream, initiating angiogenesis.

The angiogenesis acceleration model

This theory assumes that first there is up-regulation of VEGF by oncogenes. Angiogenesis is activated and proliferation of tumour cells takes place. These processes are leading to hypoxia, activating HIF expression. HIF1 alpha is up-regulated to accelerate angiogenesis and microvasculature remodeling.

Hypoxic tumour tissue may reoxygenate after treatment with radiation and some forms of chemotherapy. This effect is caused by a reduction of the oxygen consumption rate, resulting from the death of the more radiosensitive tumour cells, residing in a better oxygenated environment with higher blood supply compared to other areas of the tumour. However HIF1 alpha protein levels and also HIF1 alpha regulated proteins often increase after radiotherapy as a reason of increased free radicals after radiation. Also stress granules which are formed under hypoxia to stop protein translation and to save energy disaggregate during reoxygenation, releasing HIF1 alpha regulated transcripts under radiotherapy. Furthermore HIF1 alpha stabilization after RTX (radiotherapy) runs also via macrophage infiltration into tumours, phagocyting dying tumour cells and leading to NO (nitric oxide) production (50).

Blockade of HIF1 alpha seems to be reasonable as tumours then have no possibility to undergo anaerobic glycolysis. It reduces the proliferation rate of hypoxic cells, promotes apoptosis and necrosis which are clear mechanisms for killing of less sensitive radio- and chemotherapy resistant hypoxic tumour cells. There should be benefits when combining HIF inhibition with radiotherapy or anti-angiogenic therapies with RTX (50).

II.8. Conclusion

There are many different mechanisms leading to activation of HIF1 alpha and its signaling cascades. Modulators of the HIF besides hypoxia do exist and play an important role in resistance to radio- and chemotherapy. There are different models concerning the involvement of the HIF in the process of angiogenesis. It may initiate or accelerate angiogenesis (50), however other oncogenes up-regulating VEGF and the other angiogenic factors are involved as well, and seem even more important for tumour vasculature than the HIF. Further studies will be necessary to reveal the exact process of interaction between the HIF/VEGF/PDGF/Angiopoietins and their growth factor receptors. But blockade of activated HIF and/or angiogenesis pathways makes sense to prevent tumour proliferation and metastasis. There are many pre-clinical and clinical studies targeting HIF and angiogenesis.

Bevacizumab, a humanized monoclonal antibody, which acts by binding and neutralizing the VEGFA ligand was approved by the FDA for initial systemic treatment of patients with unresectable, locally advanced, recurrent or metastatic, non squamous NSCLC in combination with chemotherapy with carboplatin and paclitaxel (35). Some tyrosine kinase inhibitors, interfering with VEGF and PDGF receptors like Sorafenib and Sunitinib have undergone clinical phase II and III studies for non-operable NSCLC stage 3 and 4 tumours and seem to be a promising new way of anti-cancer treatment (35, 36). A synergistic effect of this agents combined with chemotherapy has been reported. However, severe side effects of these promising biological agents like pulmonary hemorrhage should be also considered. Further pharmaceutical research will be necessary in order to prevent such fatal events. A multivariate analysis identified squamous cell histology as an independent risk factor for unexpected massive and life-threatening bleedings under treatment with bevacizumab (35).

Differences between the histological subtypes of lung cancer concerning activation of signaling pathways do exist. There are preferences for certain growth factors and receptors involved in angiogenesis for each subtype of NSCLC. Therefore we should consider target tailored therapies interfering with activated intracellular molecular pathways for the individual patient and the individual tumour entity. In future the role of pathologists in cancer treatment will become even more important, providing protein expression profiles of activated signaling molecules for the individual patient, recommending potential therapy targets to oncologists and internists. Also Amir et al. (35) mentioned the release and presence of many other angiogenic factors besides VEGF, which get secreted from tumours for tumour progression. Examples for such factors are bFGF, TGF-β1, PlGF, PDGF and pleiotrophin. They propose that anti-angiogenic therapy should be tailored depending the angiogenic phenotype and expression of endothelial growth factors in each single tumour. Combination of different anti-vascular agents to target multiple pathways simultaneously may lead to additional survival benefits for patients with progressive tumours with alternating angiogenic profiles (35).

Literaturverzeichnis

1. Giaccone G. The potential of antiangiogenic therapy in non-small cell lung cancer. Clin Cancer Res. 2007 Apr 1;13(7):1961-70.
2. Parkin M TJ, Boffetta B, Samet J, Shields P, Caporaso N. World Health Organization Classification of Tumours; Pathology & Genetics; Tumours of the Lung, Pleura, Thymus and Heart. Kleihues Paul SL, editor.: IARC Press, International Agency for Research on Cancer (IARC), 69008 Lyon, France; 2004.
3. WHO. Gender in lung cancer and smoking research. 2004.
4. Guessous I, Cornuz J, Paccaud F. Lung cancer screening: current situation and perspective. Swiss Med Wkly. 2007 Jun 2;137(21-22):304-11.
5. Panani AD, Roussos C. Cytogenetic and molecular aspects of lung cancer. Cancer Lett. 2006 Jul 28;239(1):1-9. Epub 2005 Aug 19.
6. Ullmann R, Morbini P, Halbwedl I, Bongiovanni M, Gogg-Kammerer M, Papotti M, et al. Protein expression profiles in adenocarcinomas and squamous cell carcinomas of the lung generated using tissue microarrays. J Pathol. 2004 Jul;203(3):798-807.
7. Thun MJ, Lally CA, Flannery JT, Calle EE, Flanders WD, Heath CW, Jr. Cigarette smoking and changes in the histopathology of lung cancer. J Natl Cancer Inst. 1997 Nov 5;89(21):1580-6.
8. Boenisch T FA, Stead R. Handbuch II immunchemischer Färbemethoden. Naish Sally D, Dako Corporation, editor. Carpinteria, Kalifornien, USA; 1989.
9. Sebolt-Leopold JS, English JM. Mechanisms of drug inhibition of signalling molecules. Nature. 2006 May 25;441(7092):457-62.
10. Vlahovic G, Crawford J. Activation of tyrosine kinases in cancer. Oncologist. 2003;8(6):531-8.
11. Mueller MD, Vigne JL, Minchenko A, Lebovic DI, Leitman DC, Taylor RN. Regulation of vascular endothelial growth factor (VEGF) gene transcription by estrogen receptors alpha and beta. Proc Natl Acad Sci U S A. 2000 Sep 26;97(20):10972-7.
12. Voelkel NF, Vandivier RW, Tuder RM. Vascular endothelial growth factor in the lung. Am J Physiol Lung Cell Mol Physiol. 2006 Feb;290(2):L209-21.
13. Shida A, Fujioka S, Ishibashi Y, Kobayashi K, Nimura H, Mitsumori N, et al. Prognostic significance of vascular endothelial growth factor D in gastric carcinoma. World J Surg. 2005 Dec;29(12):1600-7.
14. Endo A, Nagashima K, Kurose H, Mochizuki S, Matsuda M, Mochizuki N. Sphingosine 1-phosphate induces membrane ruffling and increases motility of human umbilical vein endothelial cells via vascular endothelial growth factor receptor and CrkII. J Biol Chem. 2002 Jun 28;277(26):23747-54.
15. Matsumoto T, Claesson-Welsh L. VEGF receptor signal transduction. Sci STKE. 2001 Dec 11;2001(112):RE21.
16. Keedy VL, Sandler AB. Inhibition of angiogenesis in the treatment of non-small cell lung cancer. Cancer Sci. 2007 Dec;98(12):1825-30.
17. Chae JK, Kim I, Lim ST, Chung MJ, Kim WH, Kim HG, et al. Coadministration of angiopoietin-1 and vascular endothelial growth factor enhances collateral vascularization. Arterioscler Thromb Vasc Biol. 2000 Dec;20(12):2573-8.
18. Litz J, Krystal GW. Imatinib inhibits c-Kit-induced hypoxia-inducible factor-1alpha activity and vascular endothelial growth factor expression in small cell lung cancer cells. Mol Cancer Ther. 2006 Jun;5(6):1415-22.
19. Herbst RS, Onn A, Sandler A. Angiogenesis and lung cancer: prognostic and therapeutic implications. J Clin Oncol. 2005 May 10;23(14):3243-56.

20. Brahimi-Horn C, Mazure N, Pouyssegur J. Signalling via the hypoxia-inducible factor-1alpha requires multiple posttranslational modifications. Cell Signal. 2005 Jan;17(1):1-9.
21. Wei W, Yu XD. Hypoxia-inducible factors: Crosstalk between their protein stability and protein degradation. Cancer Lett. 2007 Nov 18;257(2):145-56.
22. Swinson DE, Jones JL, Cox G, Richardson D, Harris AL, O'Byrne KJ. Hypoxia-inducible factor-1 alpha in non small cell lung cancer: relation to growth factor, protease and apoptosis pathways. Int J Cancer. 2004 Aug 10;111(1):43-50.
23. Giatromanolaki A, Koukourakis MI, Sivridis E, Turley H, Talks K, Pezzella F, et al. Relation of hypoxia inducible factor 1 alpha and 2 alpha in operable non-small cell lung cancer to angiogenic/molecular profile of tumours and survival. Br J Cancer. 2001 Sep 14;85(6):881-90.
24. Ruas JL, Poellinger L. Hypoxia-dependent activation of HIF into a transcriptional regulator. Semin Cell Dev Biol. 2005 Aug-Oct;16(4-5):514-22.
25. Giatromanolaki A, Koukourakis MI, Sivridis E, Pastorek J, Wykoff CC, Gatter KC, et al. Expression of hypoxia-inducible carbonic anhydrase-9 relates to angiogenic pathways and independently to poor outcome in non-small cell lung cancer. Cancer Res. 2001 Nov 1;61(21):7992-8.
26. Jimenez C, Portela RA, Mellado M, Rodriguez-Frade JM, Collard J, Serrano A, et al. Role of the PI3K regulatory subunit in the control of actin organization and cell migration. J Cell Biol. 2000 Oct 16;151(2):249-62.
27. Liu J, Wu LL, Li L, Zhang L, Song ZE. Growth-promoting effect of platelet-derived growth factor on rat cardiac myocytes. Regul Pept. 2005 Apr 15;127(1-3):11-8.
28. Lokker NA, Sullivan CM, Hollenbach SJ, Israel MA, Giese NA. Platelet-derived growth factor (PDGF) autocrine signaling regulates survival and mitogenic pathways in glioblastoma cells: evidence that the novel PDGF-C and PDGF-D ligands may play a role in the development of brain tumors. Cancer Res. 2002 Jul 1;62(13):3729-35.
29. Boulven I, Palmier B, Robin P, Vacher M, Harbon S, Leiber D. Platelet-derived growth factor stimulates phospholipase C-gamma 1, extracellular signal-regulated kinase, and arachidonic acid release in rat myometrial cells: contribution to cyclic 3',5'-adenosine monophosphate production and effect on cell proliferation. Biol Reprod. 2001 Aug;65(2):496-506.
30. Fryer BH, Field J. Rho, Rac, Pak and angiogenesis: old roles and newly identified responsibilities in endothelial cells. Cancer Lett. 2005 Nov 8;229(1):13-23.
31. Chavakis E, Dimmeler S. Regulation of endothelial cell survival and apoptosis during angiogenesis. Arterioscler Thromb Vasc Biol. 2002 Jun 1;22(6):887-93.
32. Visconti RP, Richardson CD, Sato TN. Orchestration of angiogenesis and arteriovenous contribution by angiopoietins and vascular endothelial growth factor (VEGF). Proc Natl Acad Sci U S A. 2002 Jun 11;99(12):8219-24.
33. Marron MB, Hughes DP, Edge MD, Forder CL, Brindle NP. Evidence for heterotypic interaction between the receptor tyrosine kinases TIE-1 and TIE-2. J Biol Chem. 2000 Dec 15;275(50):39741-6.
34. Pufe T, Petersen WJ, Mentlein R, Tillmann BN. The role of vasculature and angiogenesis for the pathogenesis of degenerative tendons disease. Scand J Med Sci Sports. 2005 Aug;15(4):211-22.
35. Amir E, Hughes S, Blackhall F, Thatcher N, Ostoros G, Timar J, et al. Targeting blood vessels for the treatment of non-small cell lung cancer. Curr Cancer Drug Targets. 2008 Aug;8(5):392-403.
36. Goudar RK, Vlahovic G. Hypoxia, angiogenesis, and lung cancer. Curr Oncol Rep. 2008 Jul;10(4):277-82.

37. Byers LA, Heymach JV. Dual targeting of the vascular endothelial growth factor and epidermal growth factor receptor pathways: rationale and clinical applications for non-small-cell lung cancer. Clin Lung Cancer. 2007 Feb;8(Suppl 2):S79-85.
38. Cascone T, Gridelli C, Ciardiello F. Combined targeted therapies in non-small cell lung cancer: a winner strategy? Curr Opin Oncol. 2007 Mar;19(2):98-102.
39. Felip E, Santarpia M, Rosell R. Emerging drugs for non-small-cell lung cancer. Expert Opin Emerg Drugs. 2007 Sep;12(3):449-60.
40. Gridelli C, Maione P, Del Gaizo F, Colantuoni G, Guerriero C, Ferrara C, et al. Sorafenib and sunitinib in the treatment of advanced non-small cell lung cancer. Oncologist. 2007 Feb;12(2):191-200.
41. Swinson DE, O'Byrne KJ. Interactions between hypoxia and epidermal growth factor receptor in non-small-cell lung cancer. Clin Lung Cancer. 2006 Jan;7(4):250-6.
42. Vordermark D, Brown JM. Endogenous markers of tumor hypoxia predictors of clinical radiation resistance? Strahlenther Onkol. 2003 Dec;179(12):801-11.
43. Song X, Liu X, Chi W, Liu Y, Wei L, Wang X, et al. Hypoxia-induced resistance to cisplatin and doxorubicin in non-small cell lung cancer is inhibited by silencing of HIF-1alpha gene. Cancer Chemother Pharmacol. 2006 Dec;58(6):776-84.
44. Lee CH, Lee MK, Kang CD, Kim YD, Park DY, Kim JY, et al. Differential expression of hypoxia inducible factor-1 alpha and tumor cell proliferation between squamous cell carcinomas and adenocarcinomas among operable non-small cell lung carcinomas. J Korean Med Sci. 2003 Apr;18(2):196-203.
45. Hirami Y, Aoe M, Tsukuda K, Hara F, Otani Y, Koshimune R, et al. Relation of epidermal growth factor receptor, phosphorylated-Akt, and hypoxia-inducible factor-1alpha in non-small cell lung cancers. Cancer Lett. 2004 Oct 28;214(2):157-64.
46. Kim SJ, Rabbani ZN, Dewhirst MW, Vujaskovic Z, Vollmer RT, Schreiber EG, et al. Expression of HIF-1alpha, CA IX, VEGF, and MMP-9 in surgically resected non-small cell lung cancer. Lung Cancer. 2005 Sep;49(3):325-35.
47. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, et al. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res. 1999 Nov 15;59(22):5830-5.
48. Goeman JJ, van de Geer SA, de Kort F, van Houwelingen HC. A global test for groups of genes: testing association with a clinical outcome. Bioinformatics. 2004 Jan 1;20(1):93-9.
49. Polosukhin VV, Lawson WE, Milstone AP, Egunova SM, Kulipanov AG, Tchuvakin SG, et al. Association of progressive structural changes in the bronchial epithelium with subepithelial fibrous remodeling: A potential role for hypoxia. Virchows Arch. 2007 Oct;451(4):793-803. Epub 2007 Aug 3.
50. Dewhirst MW, Cao Y, Moeller B. Cycling hypoxia and free radicals regulate angiogenesis and radiotherapy response. Nat Rev Cancer. 2008 Jun;8(6):425-37.

Lebenslauf

Persönliche Daten

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Geburtsdatum:	19.06.1984
Geburtsort:	Villach
Staatsbürgerschaft:	Österreich
Familienstand:	ledig
Konfession:	röm.-kath.
Vater:	Hans Egon Eder, Hauptschullehrer
Mutter:	Margarete Eder, geb. Poschinger, Hauptschullehrerin
Geschwister:	Philipp Eder , 21 Jahre, Student FH Joanneum, Studienrichtung Fahrzeugtechnik

Schulausbildung

1990 - 1991	VS 1 Feistritz/Drau
1991 - 1994	VS Stadelbach
1994 – 1998	HS 2 Feistritz/Drau
1998 - 2003	Bundeshandelsakademie Villach; Reifeprüfung Juni 2003
2003 - 2009	Medizinische Universität Graz

Besondere Kenntnisse

Fremdsprachen:	Englisch, Italienisch, Französisch in Wort und Schrift University of Cambridge – ESOL examinations: First Certificate in English (FCE) Business English Certificate (BEC Higher)
EDV:	Maschinschreib- und Textverarbeitungskenntnisse MS Office (Word, Excel, Powerpoint) Phonotypieprüfungen WIFI Kärnten
Sonstiges:	B-Führerschein

Bisherige Arbeitsverhältnisse

Sommer 2000, 2001 und 2002	LKH Villach – Verwaltung
Sommer 2003 und 2004	Infineon Technologies – Qualitätsmanagement
Sommer 2005 und 2006	Ambulanz für Abhängigkeitserkrankungen de La Tour am LKH Villach - Sekretariat

Famulaturen

5 Wochen	Abteilung für Neurologie und Psychosomatik, LKH Villach
3 Wochen	Institut für Pathologie, LKH Villach
4 Wochen	Abteilung für Innere Medizin, LKH Villach
2 Wochen	Abteilung für Anästhesie und Intensivmedizin, LKH Villach
2 Wochen	Abteilung für Gynäkologie und Geburtshilfe, LKH Villach
4 Wochen	Abteilung für Neurologie, Ospedale Maggiore della Carità, Novara, Italien

Praktisches Jahr

5 Wochen	Hals-Nasen-Ohren-Abteilung/Allgemeine Ambulanz, Uniklinikum Graz
5 Wochen	Allgemeinmedizinfamulatur/Dr. Gerd Clement, Villach
10 Wochen	Abteilung für Urologie, LKH Leoben
10 Wochen	Abteilung für Innere Medizin, Geriatrische Gesundheitszentren Graz – Albert-Schweitzer-Klinik

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