TREATMENT OF SUBTYPES IN NON-SMALL CELL LUNG CANCER

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"Treatment of subtypes in non-small cell lung cancer"

Master literature thesis

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Abstract

Lung cancer accounts for the highest number of cancer related deaths among all cancer patients. It has been well established that exposure to tobacco significantly increases the risk of developing lung cancer. Non-small cell lung cancer (NSCLC) is diagnosed in almost 80% of all lung cancer patients. Despite different subtypes of NSCLC, treatment regimens to not distinguish between subtypes. Different stages and localization of determine treatment with surgery, radiotherapy or systemic cytotoxic agents. Adenocarcinoma, squamous cell carcinoma and large cell carcinoma are the three major subtypes make up 90% of all NSCLC patients. Differences in morphology, differentiation, smoking history and genetic alterations determine these subtypes. A new generation anti-cancer drugs have been designed to specifically target tumor-specific alterations such as amplifications or mutations. Various clinical trials have shown the importance of pretreatment screening for specific alterations that can be targeted by targeted therapies. Mutations and amplification of the epidermal growth factor have made it an interesting target for new drug development with tyrosine kinase inhibitors and mono-clonal antibodies as result. The future of NSCLC treatment will be based on individualized medicine in which molecular mechanisms underlying tumor development will be of most importance.

Subtypes in non-small cell lung cancer

In 2010, more than 220,000 new cases of lung and bronchial cancer have been diagnosed, which is about 15% of all new cancer patients in the United States of America. The estimated amount of deaths due to lung cancer is as high as 30% making lung cancer one the most lethal types of cancer after diagnosis. Differences between male and female lung cancer patients have become a lot smaller over the years. Men's mortality has reached its peak and has been steadily declining since then. Female mortality is reaching a plateau not increasing mortality after becoming the most lethal cancer type in 1985 [1]. It is well known that exposure to tobacco significantly increases the risk of developing lung carcinoma. Of the 5,000 chemicals found in tobacco smoke, over 80 have been found to be carcinogenic by causing mutations in the epithelium lining the airways [2]. Lung cancer in patients who do not smoke or exposed to other carcinogens like asbestos, are more frequently women from Asian origin. The sex difference in lung cancer could be explained by the effects of estrogens and the estrogen receptor on lung cancer pathogenesis [3]. However, it is not clear by what mechanism estrogen receptor activation affects lung cancer pathogenesis and survival [4]. Lung cancer is crudely divided into two major classes: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). About 80% of all lung cancer patients are diagnosed with NSCLC, which consists of three major subtypes. Adenocarcinoma (ADC), squamous cell carcinoma (SCC) and large cell carcinoma (LCC) make up 85% of NSCLC patients (figure 1).

Amplification and deletion in NSCLC are observed in crucial tumor suppressors and proto-ongogenes that are also often found to be mutated. Amplifications are often observed in chromosome 12, 7, 11, 1, 17 and 8. These loci contain well known proto-

oncogenes as KRAS, EGFR, ERBB2 and CDK proteins and MDM2 which are transforming when overexpressed. EGFR is next to being amplified also activated by mutations. These mutations are predominantly observed in patients who do not smoke. Suggesting the sequence of pathogenesis is different between smokers and non-smokers. Marker-proteins for subtypes of NSCLC are also overexpressed such as S100A. Import tumor suppressors are also deleted in SCLC such as $p14^{ARF}$, $p16^{INK4A}$ and FHIT [5]. p53 is rarely homozygous deleted but has been reported to be affected by loss of function mutations [6].

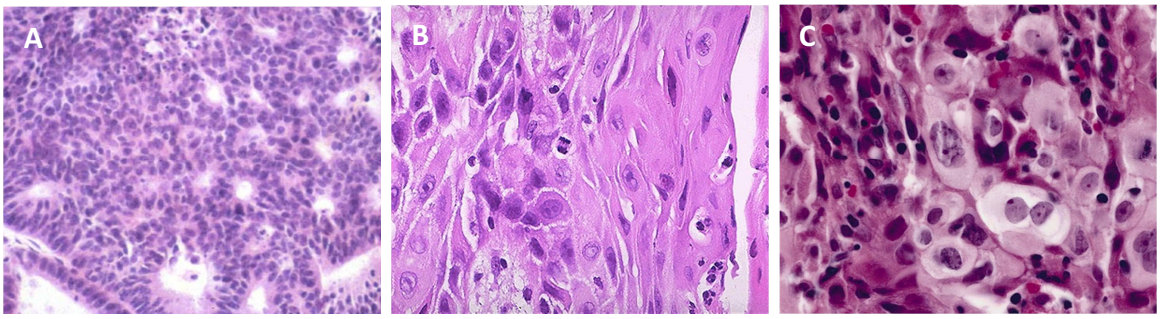

Figure 1: Histological slides of the three major subtypes of non-small cell lung cancer, **A:** adenocarcinoma, **B:** squamous cell carcinoma, **C:** large cell carcinoma

Lung carcinoma in general has been a type of cancer which has a poor survival median time of 28 months. Few prognostic factors have been identified upon diagnosis of NSCLC. Staging of the tumor is an important prognostic factor where limited disease has a more favorable outcome compared to increased staging. A second prognostic tool is the presence of any symptoms caused by the primary or secondary tumor. When patients are diagnosed without any symptoms their survival significantly increases [7]. Annual screening of a population that has an increased risk on developing lung carcinomas results in an increase of total survival with 12% compared to all cancers detected in ELCAP. The presence of specific genetic

alterations can also affect the survival and recurrence of resected tumors. Expression of single tumor suppressors such as pRb, p16, p21 and p27 have a beneficial effect on survival. Mutations in p53, expression of FGF and VEGF however, result in a shortened survival time.

Adenocarcinoma

Adenocarcinomas (ADC) are the most frequent type of lung tumor in the peripheral airways which is qualified by the WHO as "a malignant epithelial tumour with glandular differentiation or mucin production, showing acinar, papillary, bronchioalveolar or solid with mucin growth patterns or a mixture of these patterns". Histological analysis shows a distinct morphogenesis of the cells that can form duct like structures. Immunohistological staining of adenocarcinomas are positive for mucin and CK7 but negative for CK20. Cytokeratin staining can distinguish pulmonary from nonpulmonary adenocarcinomas. Cytokeratin 7 is mainly expressed in pulmonary tissue whereas CK20 expression is restricted to gastrointestinal and urothelium. This characteristic makes CK20 a useful marker to distinguish between a primary lung adenocarcinoma or metastatic colon adenocarcinoma [8]. A relative novel immunohistochemical marker thyroid transcription factor 1 (TTF-1) has been shown to be exclusively expressed in 75% of all adenocarcinomas. TTF-1 is expressed during embryogenesis in the developing thyroid tissue and in the branching bronchus. After development, expression remains in the alveolar epithelial type II cells. This suggests a role of TTF-1 in lung endothelial development [9, 10]. When the tumor is TTF-1 positive, a thyroid metastasis can be excluded by an absent expression of thyroglobulin [11, 12].

ADC cells are usually found as single cells or in three-dimensional clusters defined by their sharp lineation. The luminous cytoplasm is usually present in large quantities

that appears homogenous and can show a granular or foamy morphology. Mucin presence is usually limited to one single vacuole, however, multiple vacuoles are not uncommon (figure 1a). Well differentiated ADC is characterized by one well-shaped nucleus without much abnormality. Irregularities in the nucleus increases when the tumor has a decreased differentiation status. ADC is hard to distinguish from bronchioloalveolar carcinoma (BAC) because of man overlapping cellular features. Moreover, in about 40% of ADC patients BAC components make up more than 10% of the primary ADC tumor. It is thought that atypical adenomatous hyperplasia (AAH) is a precursor lesion that can develop in both BAC and ADC. BAC lesions can give rise to the development of ADC. AAH and BAC have a similar growth pattern by replacing the endothelium that lines the peripheral airways. There is no clear definition when an AAH lesion consists of BAC components. However, the presence of AAH lesions in both a BAC and ADC background suggests that there is a sequential pattern of pathogenesis in ADC development [13].

ADC is characterized by five distinct patterns of growth and spread. The most prevalent site of tumor development is the peripheral airways in combination with pleural puckering. Also, a combination with V-shaped desmoplastic fibrosis is often observed with poor lining of the tumor. Secondly, ADC also develops in central or endobronchial airways that can result in bronchial obstruction. A third growing pattern is characterized by diffuse tumor growth throughout the entire lung and is typical for the formation of mucinous BAC. Notably, the original structure of the airways is preserved. Diffuse bilateral spreading of the tumor in combination with the formation of tumor nodules is characteristic for the fourth growing pattern. Finally, ADC can develop in the background of fibrosis and scar formation that can mask the tumor development in imaging methods [14, 15].

ADC is subtype of lung carcinomas in which patients that have a smoking history are not overrepresented. Moreover, the relative risk of developing ADC has the lowest increase of all the major subtypes in the smoking population [16]. Interestingly, there are differences in the genetic alterations between patients who have a history in smoking and patients who never smoked. KRAS mutations in exon 12 are observed in 30-40% of patients with a smoking history [17]. These mutations have been reported to have prognostic value concerning the survival of patients. Numerous survival analyses have been performed on lung cancer patients with and without KRAS mutations. One would expect that a mutation in the KRAS gene promotes tumor development and therefor have a negative prognostic factor. However, upon spreading of the tumor, the KRAS mutant patient group seems to have an increased survival. When the tumor is diagnosed without any lymph node metastases, no differences in survival are detected [17]. Notably, the presence of KRAS mutations in plasma does not seem to be a prognostic factor in patients with advanced NSCLC [18]. These differences in survival might be caused by changed and improved treatment regiments. Also, patient group selection, differences in samples and analysis can be causing differences in survival. Additional mutations have been observed in p53, STK11 and EGFR at a frequency of 70%-30%. Mutations found at a frequency of 20% in known tumor suppressors NF1 and LRP1B were not annotated in ADC before [19]. In addition to breast carcinoma, a segment of ADC also expresses the HER2/neu receptor which is the target of Herceptin used to treat HER2/neu positive breast carcinoma [20]. ADC is also characteristic for it retained $p27^{KIP1}$ expression that results into an increased survival in comparison with tumors that have lost the expression of this protein [21]. Further genetic alterations observed in ADC are large-scale amplifications of chromosomes 1q, 5p and 8q. Along with these amplifications are deletions that 9p, 8p, 18q, 19p and 15q. Chromosomal region 9p contains the gene

encoding for the tumor suppressor p16, a protein that slows down proliferation by inhibiting the formation of Cdk/Cyclin complexes. PTEN, which dephosphorylates PIP_3 thereby inhibiting PI3K signaling, is hemizygously deleted in a fraction of ADC as well [22]. Gain of chromosome 1q and loss of 8p have been related to tumor progression and the development of metastases. Surprisingly, the amount of genomic deletions proved to be more abundant in the primary tumor whereas gain of chromosomes was more often observed in metastases. This suggests that tumor development requires the deletion of tumor suppressors while the formation of a metastases prone tumor requires increased expression of certain genes [23].

Gene expression profiling of ADC samples revealed multiple subclasses within the dataset using mRNA expression followed by hierarchical clustering. These subclasses seem to be defined by the level of differentiation and the expression of multiple markers. An unpredicted value of the expression profiling was the identification of distant metastases to the lung. The expression pattern of this cluster suggested that the primary tumor was a colon carcinoma. Neuroendocrine gene expression and alveolar pneumocyte marker proteins determine the two largest clusters out of four. Expression profiles also have a prognostic survival value which has been validated in later studies. Neuroendocrine expression is associated with a 50% decrease in median survival-time in all stages of diagnosis. Patients with expression of pneumocyte marker proteins had a favorable outcome compared to the combined survival of the other clusters. Exposure to tobacco seems to have a limited effect on gene expression. The cluster characterized by expression of pneumocyte markers had a 50% fewer pack-years compared to the entire dataset [24].

Squamous cell carcinoma

Squamous cell carcinoma (SCC) is tumor characterized by keratinization of the epithelium of which the carcinoma arises and is more prevalent in patients who have a history in smoking. SCC is defined by the WHO as "a malignant epithelial tumour showing keratinization and/or intercellular bridges that arises from bronchial epithelium". The presence keratinization and intercellular bridges in SCC is correlated to their differentiation status. SCC biopsies are characterized by single cells in a background of necrosis and cellular debris. Tumor cells are large and have irregular hyperchromatic nuclei and unusual morphology demonstrated by their spindle or tad pole-like appearance (figure 1b). Undifferentiated tumor cells are hard to distinguish from large cell carcinoma and often the presence of keratin leads to the diagnosis of SCC [14, 15]. Immunohistochemical analysis shows expression of CK5, CK6 and CK7 where CK20 expression is negative. This expression pattern is similar to other subtypes of NSCLC. Unlike ADC, TTF-1 expression is rarely observed in SCC, suggesting a different cellular origin of SCC. Expression of p63 however is specific for SCC and distinguishes SCC from the other subtypes [25, 26]. Also, expression of carcinoembryonic antigen (CEA) is observed in SCC. CEA is an adhesion molecule which is only expressed during embryogenesis and is lost before birth. Expression of CEA is exclusively observed in smokers and is suggested to be a chemoattractant for neutrophils [27].

SCC is a subtype in which patients who have a history in smoking are overrepresented, which could explain the expression of CEA. A history in smoking can increase the risk of developing SCC over 10-fold compared to never-smokers. Current smokers have an increased risk that as high as 25 times more than never-smokers. There is also a clear dose-effect relationship in the amount of cigarettes and

the increased risk of developing SCC, more so than in other subtypes of lung carcinoma [16]. The exposure to tobacco creates a specific gene expression signature in bronchial epithelium with up-regulated genes involved in oxidative stress response, anti-apoptosis, proliferation and adhesion. Down-regulated genes are thought to have a function in cellular differentiation, growth repression and inflammation. In SCC cells this signature is maintained and even enhanced [28]. Immunohistochemistry showed expression of p63, which is often amplified in SCC. p63 functions as on oncogene by inhibiting the expression of pro-apoptotic proteins Puma and Noxa [29, 30]. p63 is located on the chromosome 3q, a region often amplified in SCC. Other important genes in this region are PIK3CA and ECT2. PIK3CA is a subunit of the PI3K that activates the PIP_3 pathway, whereas ECT2 plays a critical role in cytokinesis but fails to transform fibroblasts [31, 32]. Other amplified regions are 12p, 5p, 8q24 and 2p15 that contain genes like Myc, KRAS and Bcl family genes. Regions that are often deleted are 3p, 4q, 5q and 9p. There is also a difference in genomic aberrations between primary SCC and lymph node or distant metastases. Gain of chromosomal regions 2p and 8q correspond with deletion of 8p, 11q and 13q21. Gain of chromosome 8q has been described earlier in other cancer types in relation to a poor prognosis and metastatic progression [33]. Focal amplification in SCC is often observed at locus 7p12, the location of the endothelial growth factor receptor. Different results have been published in regard to whether EGFR expression in NSCLC is limited to SCC alone. EGFR overexpression at the plasma membrane drives proliferation by activating PI3K and Ras [34]. This receptor has been a target of novel drug development.

About 70% of SCC develops in the main bronchi in the airways from bronchial epithelium. The other 30% of SCC is diagnosed in the peripheral airways. There is

evidence that the carcinogenic process differs between the locations in which SCC develops. Also, SCC that develops in the peripheral airways is more frequently differentiated. Despite the differences in differentiation status there is no difference in the survival rate of peripheral- and central-SCC [35]. SCC is compared to other subtypes difficult to surgically remove because of an increased risk of both local and systemic recurrence [36]. However, SCC gives rise to less lymph node and distant metastases. Metastases that do form are usually found localized in the brain, bone or in the mediastinum [37].

Large cell carcinoma

Large cell carcinoma (LCC) is the smallest subtype of NSCLC that will be described in this thesis and makes up 9% of all diagnosed NSCLC. LCC is a subtype that is poorly differentiated and lacks morphological characteristics of ADC, SCC or SCLC. The WHO defines LCC as: "Large cell carcinoma is an undifferentiated NSCLC that lacks the cytologic and archictural features of small cell carcinoma and glandular or squamous differentiation". Histological diagnosis is usually made by exclusion of adenomatous or squamous elements such as duct formation or keratinization respectively. Cytologic samples are usually filled with cellular aggregates and borders are ill defined with a high nuclear to cytoplasmic ratio. Nuclear shapes vary from round to extremely irregular dominating the morphology of the cell (figure 1c). Immunohistochemistry is of minor value in LCC because of the absence of useful and specific markers. Large cell neuroendocrine carcinoma (LCNC), a major subtype of LCC that makes up 30% of all LCC cases, expresses TTF-1. Cytokeratin expression is usually negative in LCC and LCNC [38].

As in the majority of subtypes in lung cancer, patients with a smoking history are predominant in LCC. Location of tumor development is often observed in the peripheral airways where it is usually detected as a sphere using radiodiagnostics. Invasive behavior has been reported mainly into the visceral pleura and chest wall. Surgical resection reveals a pink colored tumor with a necrotic core [14]. The location metastasis development is similar to that of other NSCLC subtypes. Attempts to create a gene expression profile have been unsuccessful because of the lack of consistent up- or down-regulation of a cluster of genes [39]. This can be explained by the high degree of aneuploidy present in LCC. Little is known about the copy number alterations in LCC, there is however data of LCNC. Deletion of 3p, 4, 5q and 13 are observed together with gain of region 5p. These deletions and amplification however are not specific for LCNC and are observed in other subtypes of lung carcinoma, including small cell lung cancer [40]. Despite their high degree of genomic instability, a high percentage of LCNC is prone to apoptotic behavior. In a relative high percentage of cells, the apoptotic pathway initiated by p53 and Bax expression is intact. Over 50% of tested LCNC samples showed a nuclear localization of p53. Bcl2 negative samples, an anti-apoptotic protein, were Bax positive, which promotes apoptosis. However, no mutational analysis was performed on the p53 positive cells [41]. Research performed later showed that only 20% of all LCNC had an unaltered *tp53* gene region. Mutations (15%), loss of heterozygosity (20%) and a combination of the two (45%) could potentially disrupt the function of p53. Additional tumor suppressor alterations were found in Rb in the form of either absence of the protein or a mutation inactivating the Rb protein [42].

Diagnosis, staging and survival of lung cancer

In up to 50% of all patients, initial indications of lung cancer are often related to respiratory symptoms like coughing, dyspnea, and chest pain. However, respiratory symptoms like coughing are rarely associated with lung cancer by general practitioners. Moreover, hemoptysis is more often explained by other nonthreatening diagnoses than lung cancer [43]. In addition to symptoms relating to a possible pulmonary lesion, information on the general condition is required to estimate if the patient is eligible for surgery or other treatment modalities. Presence of any additional conditions may also affect the outcome of possible treatment options. Respiratory symptoms are usually caused by the primary lesion whereas nonspecific systemic symptoms are likely to be caused by the formation of metastases. Symptoms such as weight loss, fatigue, fever, clubbing of the fingers and general weakness are non-site specific symptoms that could be caused by a wide variety of pathophysiological mechanisms. Metastases that cause site specific symptoms give rise to symptoms associated with the tissue in which the metastasis is developing. Common sites of lung cancer metastases are the bone marrow, liver, kidneys and brains. Serum marker levels can be measured to determine the possibility of distant metastases. Serum calcium levels increase upon the release of phosphate-calcium caused by the growth of bone metastases. Sensitivity of this marker however is low and only detects extensive metastases in bone tissue. Decreased sodium serum levels can point to a paraneoplastic syndrome caused by tumor anti diuretic hormone secretion which leads to water retaining [44]. A bone scintigraphy or PET scan is used to localize the formation of bone metastases whereas a MRI is more suitable to detect brain metastases [45].

Upon suspicion of lung cancer in a patient, physical examination should be focused on a possible enlargement of lymph nodes located in the neck, supraclavicular and in the arm pits, differences in percussion or auscultation of the lungs and the enlargement of the liver. Lymph node enlargement and increased size of the liver could be an indication for the development of metastases [46]. In addition to the physical examination, a chest x-ray is essential in the examination of lung cancer. Interestingly, radiography shows a biased location increasing towards the right lung and the upper lobe of the lung [47]. Also, in an asymptomatic patient displaying a lesion is an indication of lung cancer. However, only 5% of all lung cancer patients are diagnosed in the absence of typical lung cancer symptoms [45].

A bronchoscopy is implemented after a positive chest x-ray to confirm the presence of the lesion and to obtain histological or cytological proof of the tumor. In order to make a histological diagnosis, four to five sample tissues are required to obtain a specificity of more than 90% [48]. A bronchoscopy also allows for the assessment of endobronchial spread of the carcinoma. This information is essential in grading the tumor and to determine the type of surgical resection that might be performed. When the diagnosis of lung cancer is made, all patients are imaged using a chest CT-scan. Despite the low sensitivity and specificity in determining tumor size, invasiveness behavior and the presence of metastases in the mediastinum, a CT-scan is made to select enlarged lymph nodes that possibly hold malignancies. A whole body CT-scan can also be used to detect distant metastases, however, the sensitivity of this method is low [49] and therefore actually never obtained. The use of a FDG-PET scan significantly increases the sensitivity and specificity of detecting both mediastinum and distant metastases. Also, the FDG uptake by the primary tumor is reversely correlated with the prognosis based on tumor size [50]. However, both CT and

FDG-PET imaging methods do not have similar sensitivity and specificity compared to a mediastinoscopy, which is still the golden standard in determining the presence of mediastinal lymph node metastases. At least 5 lymph nodes are required to obtain both a high specificity and sensitivity. A cervical mediastinoscopy provides access to lymph nodes 1, 2R, 2L, 4R, 4L and 7. Lymph nodes 5 and 6 are accessible by means of a parasternal mediastinoscopy. The obtained lymph nodes are histologically analyzed to determine the presence of malignant cells. Depending on the localization of the primary tumor different lymph nodes need to be resected. When a tumor is localized in the right lung or the lower left lobe, lymph nodes 2R, 2L, 4R, 4L and 7 should be removed and analyzed. Biopsy of lymph nodes 5 and 6 should be performed when the tumor is localized in the upper lobes of the left lung. Performing a mediastinoscopy in the presence of distant metastases is not informative and should therefore not be performed. Subsequently to positive lymph nodes in a FDG-PET a mediastinoscopy should be applied to obtain a tissue confirmation. Positive lymph nodes and nonspecific symptoms such as weight loss and general misery give rise to the suspicion of distant metastases [51]. Recent developments in imaging techniques have provided an approach to reduce the number of mediastinoscopies to confirm tumor staging. Transesophageal ultrasound–guided fine-needle aspiration (EUS-FNA) allows non-invasive examination of lymph nodes in the mediastinum close to the esophagus (figure 1). Lymph nodes can be localized using echography and cytologic samples are obtained using a 22-gauge needle to assess the presence of malignant cells [52]. EUS-FNA has a relative low sensitivity of 69% and a negative predictive value of 88%. Because of this low sensitivity, EUS-FNA is not sufficient to fully replace a mediastinoscopy. However, a combination of EUS-FNA with endobronchial ultrasound-guided fineneedle aspiration (EBUS-FNA) in which lymph nodes in vicinity of the trachea and main bronchi can be examined greatly increases the sensitivity to

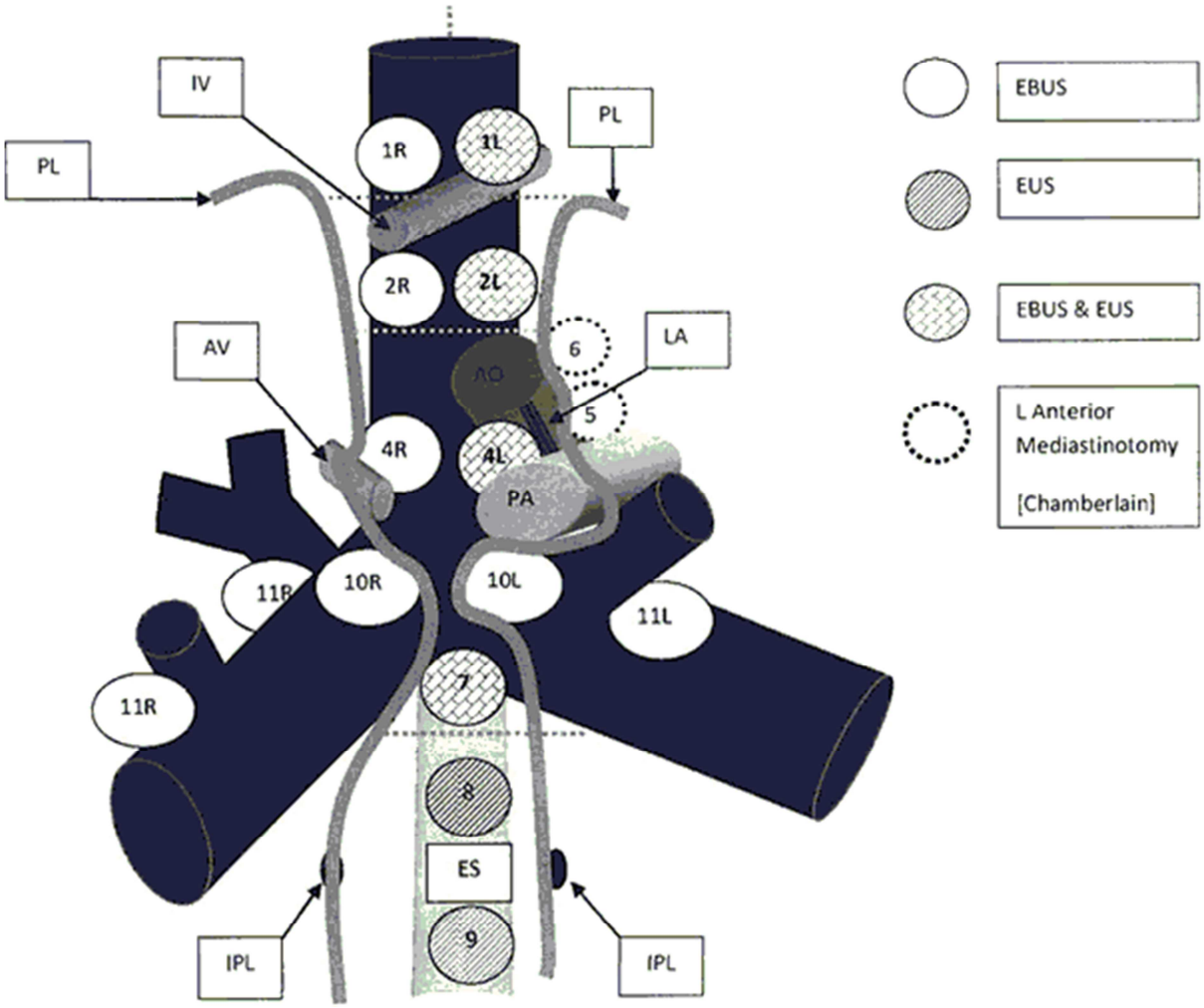

Figure 2: Schematic representation of lymph nodes accessible by EBUS, EUS, and left anterior mediastinotomy: (Chamberlain procedure). **EBUS**: endobronchial ultrasound, **EUS**: endoscopic ultrasound, **IV**: innominate vein, **AV**: azygos vein, **PL**: pleura, **AO**: aorta, **PA**: pulmonary artery, **ES**: esophagus, **IPL**: inferior pulmonary ligament, **LA**: ligamentum arteriosum. Numbers represent lymph node stations according to Mountain and Dressler [53].

Stage level	T	N	M	% patients
Ia	T1a, b	N0	M0	15
Ib	T2a	N0	M0	13
IIa	T1a, b	N1	M0	2
	T2a	N1		4
	T2b	N0		4
IIb	T2b	N1	M0	2
	T3	N0		14
IIIa	T1-3	N2	M0	20
	T3	N1		6
	T4	N0,1		2
IIIb	T4	N2	M0	1
	T1-4	N3		3
IV	T_{any}	N_{any}	M1a, b	14

Table 1: Tumor staging according to the 7th edition of the IASLC. **T**: tumor size and invasive behavior, **N**: lymph node metastases, **M**: metastatic tumor growth [54].

93% together with a negative predictive value of 97% (figure 2) [55]. However, there is still discussion whether this trial was performed without proper design and methods [56, 57]. Additional randomized prospective studies are required to confirm the importance of combining both methods to replace the invasive mediastinoscopy.

The final diagnosis of all tumors is expressed in the TNM classification. Recently the IASLC has revised the staging of lung cancer. The new classification is shown in table 1. 'T' describes the tumor size and the invasiveness of the tumor where T1 is a tumor smaller than 3 cm in diameter. A T3 tumor is bigger than 7 cm in diameter or displays invasive growth in adjacent structures. Invasive growth into structures such as the heart, aorta or laryngeal nerve is characterized as a T4 tumor. The 'N' classification provides information on the presence of any lymph node metastases. The higher the

N stage the more central lymph nodes are contaminated with malignant cells. 'M' status defines the presence of detectable metastases. Secondary tumors in the same lung and distant metastases are classified as metastases and the presence of additional micrometastases is very likely[54] . Staging of the tumor determines further treatment and is the most important prognostic factor. Survival is significantly higher in patients that have been diagnosed with Ia NSCLC. Unfortunately, only 15% of all patients are diagnosed in this stadium of tumor development. Survival of patients stratified by their tumor stage at point of diagnosis is shown in figure 2. Patients who are diagnosed with stage Ia NSCLS have a fair diagnosis and a 5-year survival of 60% whereas the 5-year survival of stage IV patients is only 1%. 1-year survival also clearly shows a trend in staging [58]. This data strongly suggests that survival rates can be improved significantly when NSCLC is detected in an earlier stage of disease. Multiple trials have been performed or are underway to study the effects of lung cancer screening in populations more susceptible to develop NSCLC. In a recent trial, the 10-year survival of patients who were diagnosed with stage I NSCLC by means of annual screening was as high as 92%. This confirms that early detection significantly increases survival over improved treatment regimens [59].

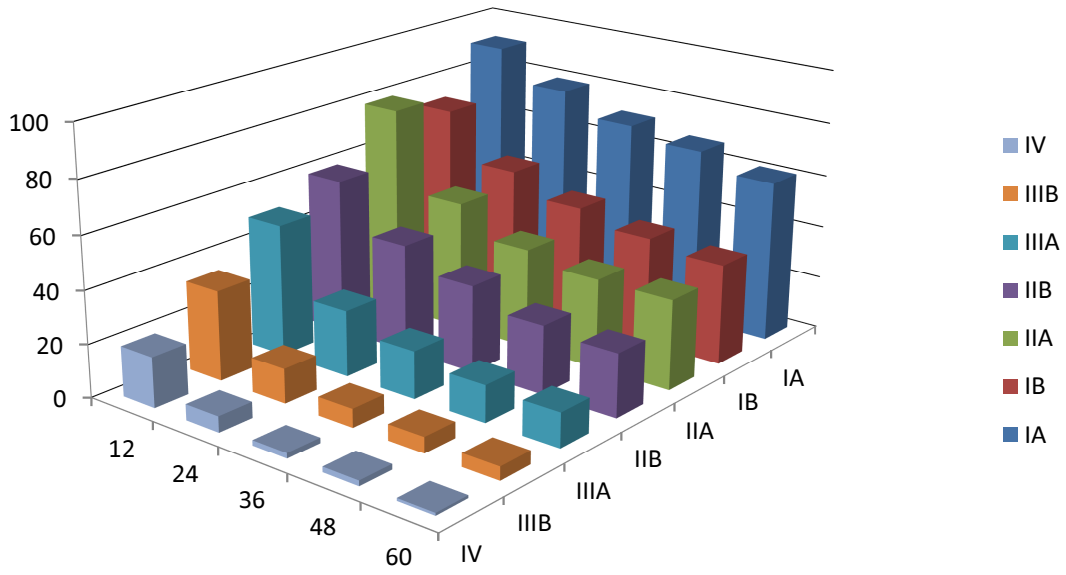

Figure 3: Survival of NSCLC patients (y-axis) over time (x-axis) stratified to their stage upon diagnosis (z-axis) [58].

Conventional treatment of non-small cell lung cancer

Conventional treatment of NSCLC depends on the staging of the tumor at the time of diagnosis. Current treatment regimens do not distinguish between the different subtypes of NSCLC or specific genetic alterations such as amplifications, deletions or mutation status except for treatment with EGFR receptor inhibition. Local NSCLC up to stage IIIA can be surgically removed when the location and spread of the tumor allows resection. According to Dutch guidelines, three parameters are crucial in determining the eligibility of the patient for surgical resection of the tumor. Perhaps the most important factor is determining if the resection has a positive effect on the outcome of the disease. In addition, the physical condition of a patient must be able to sustain the surgery. Finally, when the tumor is to be removed surgically, additional tissue samples are obtained through mediastinal lymph node analysis. When the tumor is staged in T1 or T2 surgical resection has a positive effect of the outcome of the disease. Tumors are removed by means of a lobectomy and not local removal of the tumor. Local removal results in an increase of local and systemic recurrence of the tumor and has therefor a lower survival rate over 5-years [60]. When the primary tumor has grown into multiple lobes of one lung or the location of the lesion is central, a bilobectomy or a pneumectomy can be considered. It must be noted that mortality of a pneumectomy is 3-fold higher compared to a lobectomy. T3 stage tumors that display invasive growth into the inner thorax lining or the diaphragm can be helped with surgery as well. Depending on the tumor stage survival of surgically removed NSCLC patients remains poor [44].

Adjuvant therapies have been proven useful in prolonging survival of resected NSCLC patients. Trials that introduced preoperative chemotherapy treatment showed

significant increase in survival. These trials were ended prematurely because of the strong effect of the chemotherapy treatment that withholding chemotherapy would be in conflict with ethical codes [61]. In addition to chemotherapy, postoperative radiotherapy has also proven to be successful. Local recurrence of the tumor decreased significantly and therefore the survival and cure rate increased in these patients [62]. Because of the positive effects of radiotherapy and chemotherapy, a combination of both therapies might have an additional effect on survival and disease progression. Patients who were diagnosed with inoperable NSCLC in stage I, II or III were subjected to radiotherapy alone or radiotherapy with different dose and frequency of chemotherapy. Additional chemotherapy improved both disease progression and survival of all patients [63].

Stage IIIB (lymph node metastases) and IV (distant metastases) are treated with a double or triple agent combination therapy. No major differences have been identified between different agents and combinations used during treatment. Treatment combinations are generally made out of platinum containing agents, topoisomerase II inhibitors, alkylating agents and taxols. Depending on the geographic location treatments will be different in mainly the platinum-based agents. American oncologists prefer treatment with carboplatin, also named paraplatin, because of its reduced adverse effects compared to cisplatin. Treatment in Europe is mainly based on cisplatin because of the relative harmless side effects such as nausea. More severe side effects are mainly neurotoxicity resulting in loss of touch. Cisplatin has the advantage that it does not affect the hematopoietic synthesis of platelets and erythrocytes. Current medicine is improved in such a way that side effects of cisplatin are more controllable making it the platinum agent of choice in Europe. Both carboplatin and cisplatin have a similar mechanism of action since they

both contain platinum in their molecular structure. Platinated agents enter the cell after intravenous administration where they are metabolized into aquated platinum exchanging their chloride ions for water molecules. This exchange brings about a conversion in charge from negative to positive. This positive charge allows the interaction with DNA, RNA and proteins within the nucleus and leads to the crosslinking causing DNA damage and crosslinking products of RNA-DNA and protein-DNA. A sufficient amount of DNA damage will lead to either apoptosis or necrosis in the absence of a proper functioning DNA damage repair system [64]. Cisplatin has also been shown to induce apoptosis in a DNA independent manner. The induction of endoplasmic reticulum leads to the activation of essential caspases 3 and 12 that activate the apoptosis pathway. This pathway has not been reported to be activated by other platinated agents [65].

In addition to cisplatin, etoposide is often administered in various combination therapies. Etoposide is a topoisomerase II inhibitor that has some tumor selectivity. Proliferating cells express topoisomerase II to a higher level compared to normal cells. Topoisomerase II cleaves the DNA backbone in order to reduce mechanical stress induced by the winding of DNA during the replication step. Inhibition of topoisomerase II by etoposide results in double and single stranded DNA breaks [66, 67]. Doxorubicin also inhibits the function of topoisomerase II using a different mechanism of action. In contrast to etoposide, doxorubicin interacts with the DNA directly affecting its structure.

Next to DNA-damaging agents, other mechanisms of cell death are also inducible using a different type of agent. Taxanes inhibit the formation of microtubules by interacting with the free GDP-bound tubulin. By sequestering GDP-bound tubulin, microtubule elongation is inhibited. Microtubule elongation is an essential process in

mitosis and has therefor a stronger effect on rapidly proliferating cells. Treatment with paclitaxel prevents the proper formation of the mitotic spindle and introduces mitotic arrest which leads to mitotic catastrophe eventually.

Despite a wide variety of cytotoxic agents and different combination therapies, NSCLC patients have a high incidence of acquired resistance and non-responsiveness to first line treatment. In NSCLC cell lines multiple proteins have been identified that cause multidrug resistance in patients. Multidrug resistance protein I (MRP1) and the lung resistance protein (LRP) are transport proteins that are able to dispose cytotoxic agents from the intracellular environment. MRP1 is a family member of the ABC transport proteins and has been associated with drug resistance in breast and gastric cancer in addition to lung cancer [68]. LRP expression *in vitro* gives rise to resistance against cytotoxic drugs such as etoposide and doxorubicin [69]. In addition to transporter molecules, upregulation of excision repair cross-complementing 1 (ERCC1) leads to resistance against platinated cytotoxic agents such as cisplatin and carboplatin. ERCC1 is a key enzyme in the nucleotide excision repair mechanism that facilitates the removal of platinum-DNA products thereby preventing the formation of single- and double stranded DNA breaks [70]. In a prospective study, researchers showed the impact of expression of proteins related to the response to first line cytotoxic agent treatment. High expression of MRP1 resulted in a decreased response to first line treatment and on tumor-free survival. Similar results were obtained for LRP and ERRC1 expression, a lower expression resulted in increased response and increase overall survival [71].

Second generation treatment of non-small cell lung cancer: targeted therapies

The new generation in cancer treatment has been developed in a different manner compared to the first generation of cytotoxic agents. Rational development has revealed more tumor specific targets to decrease side effects and increase treatment efficacy. One of the most successful targets in NSCLC treatment has been the EGFR receptor. Both antibodies and small molecule inhibitors have been developed to inhibit the proliferation pathway induced by EGFR activation. A second treatment has proven to be effective in multiple trials targets the vascular endothelial growth factor (VEGF) to prevent angiogenesis and formation of new vasculature that promotes tumor growth. Development of the second generation targeted therapies and genetic screening to assess treatment response are becoming more important in the perspective of treatment individualization.

Epidermal growth factor receptor

Epidermal growth factor receptor 1 (EGFR) is part of the EGFR family made out of EGFR 1-4. These transmembrane receptors are activated upon ligand binding to their extracellular domain followed by hetero- or homodimerization. Subsequent to dimerization of activated receptors, the intracellular kinase domain cross-phosphorylate tyrosine residues on which results in a conformational change revealing docking sites that facilitate the activation of downstream effectors In NSCLC, EGFR1 has been identified as a major driver proto-oncogene in mainly in the adenocarcinoma subtype. Adenocarcinoma patients have been reported to have an activating mutation rate of 20% which makes up for 60% of all NSCLC EGFR mutations

[72]. Most reported mutations are in exon 21 on residue 858 where thymidine is substituted for a guanine resulting in the exchange of leucine for an arginine. In addition to exon 21 mutations, deletion of exon 19 with residues 746 to 750 has been reported [73]. Mutations in EGFR have been reported to constitutively activate the receptor that leads to activation of the PI3K and MEK pathway contributing to the uncontrolled proliferation of tumor cells. Interestingly, it seems that mutations in EGFR are mutually exclusive with KRAS mutations, which could be explained that KRAS is downstream of EGFR activation [74]. Activation of EGFR has been proposed to be caused by structural alterations that inhibit the allosteric autoinhibition of the inactive receptor [75]. Small molecule inhibitors and humanized antibodies have been designed to inhibit the activation of the EGFR receptor thereby reducing proliferation and making tumor cells more susceptible to apoptosis caused by adjuvant therapies. In current NSCLC treatment, two EGFR specific small molecule inhibitors are given as second line treatment in EGFR mutation or amplification positive patients. Both erlotinib and gefitinib bind to the adenosine triphosphate binding site of the EGFR in a reversible manner. Compared to the wildtype EGFR, mutations in EGFR cause the binding affinity for ATP to decrease and increases the affinity for the small molecule inhibitors resulting in a decrease in cross-phosphorylation and thus to the inhibition of downstream effector proteins [76].

In one of the first reports on specific EGFR inhibition, small molecule inhibitors specifically inhibited EGFR phosphorylation at nanomolar concentrations. Cell lines that overexpressed EGFR showed a significant decrease in proliferation when treated with the small molecule inhibitor [77]. Surprisingly, the first phase III clinical trials of gefitinib and erlotinib did not show increased survival when patients were receiving EGFR inhibitors in addition to their standard chemotherapy. Initial thoughts were the

dosage of the tyrosine kinase inhibitors was not high enough to have a clinical effect. Interestingly, difference in survival was observed in patients without a history in smoking (figure 3) [78, 79]. However, a trial that selected their patients based on their EGFR mutation status revealed a strong effect of EGFR inhibitors on the survival and disease progression in patients who had an activating EGFR mutation [80]. Additional trials revealed a great beneficial effect when patients are selected for treatment with EGFR inhibitors as a first line agent. Survival of patients increased two-fold compared to non-selected NSCLC patient groups. In addition to EGFR mutation status, KRAS mutations have been shown to be a negative prognostic factor for erlotinib and gefitinib response. As described above, KRAS is downstream oncogene activated by EGFR thus inhibiting EGFR activation does not result in the inactivation of KRAS [81]. A different improvement of small molecule inhibitors against the EGFR receptors are the relative moderate adverse effects compared to conventional chemotherapy. Less than 20% of all gefitinib patients suffered from grade three side effects, which can be as high as 75% in conventional regimens [82].

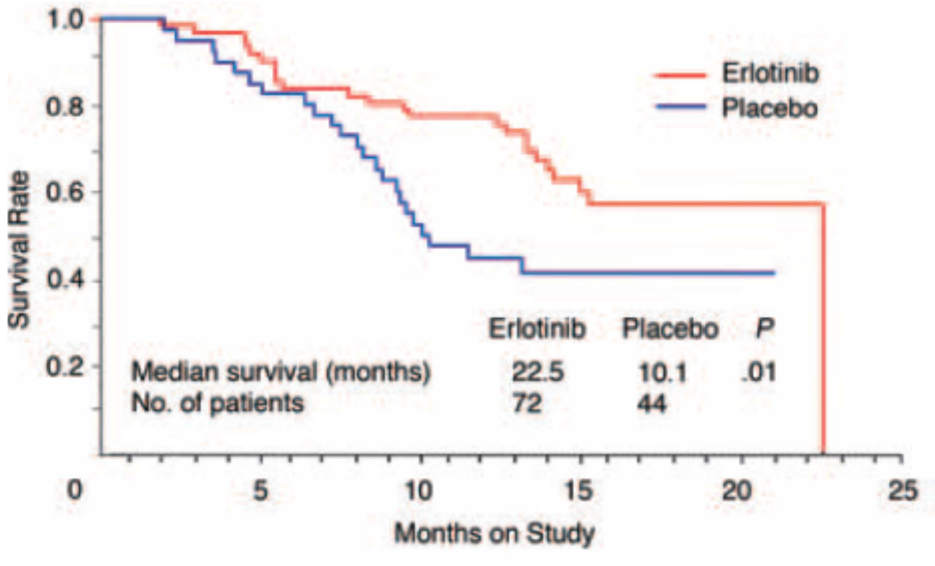

Figure 3: Survival of non-smoking patients treated with the EGFR tyrosine kinase inhibitor erlotinib [79]

Next to small molecule inhibitors, a humanized antibody against EGFR has also been developed and proved to be successful in treatment of metastatic colorectal and head- and neck cancer. Cetuximab is a monoclonal antibody that binds to the EGFR receptor thereby inhibiting the binding of ligands such as EGF and TNF-α. Preventing this interaction inhibits the activation of EGFR and downstream effectors described above. In addition to EGFR activation inhibition, tumor cells are marked with antibodies that can select the cells for apoptosis induced by the host immune system. Both antibody-dependent cellular cytotoxic and the complement system contribute to the specific clearance of EGFR expressing tumor cells [83, 84]. As observed earlier in NSCLC treatment with small molecule inhibitors of EGFR, no differences in progression free and overall survival were observed in initial trials with cetuximab [85]. However, when patients are selected for EGFR expression, there is a modest effect of cetuximab on the survival and progression free survival. All subgroups participating had a survival increase of one month, however, adenocarcinoma

patients has an increase of two months with cetuximab treatment. The additional adenocarcinoma can be contributed to the higher level of EGFR expression and mutations in this histological subtype. Increased survival and response to cetuximab does not go with an increase in severe adverse effects. A notable adverse effect is the formation of an acneiform rash in addition to increased infusion reactions and a hypomagnesemia [86]. In addition to EGFR expression a second predictive biomarker has been identified to exclude patients who will not respond to cetuximab treatment.

Unfortunately, resistance mechanisms in recurrent NSCLC patients have been identified to both targeted therapies against EGFR mutation or overexpression. In patients who had a recurrent disease after erlotinib or gefitinib treatment a mutation was identified at residue 790 from a threonine to a methionine. This mutation is identified in 50% of all patients who acquired resistance as well as in patients who were resistant prior to treatment with tyrosine kinase inhibitors [87]. Residue T790 in EGFR is a key residue in the binding of ATP resulting in the activation of the receptor. The exchange of threonine for methionine results in an increase of ATP affinity for the ATP binding pocket of EGFR. The binding of ATP competes with the binding of small molecule inhibitors erlotinib and gefitinib resulting in the activation of the receptor restoring its oncogenic characteristics [76]. A second mechanism of resistance observed in targeted therapy is the amplification of the *MET* oncogene, an amplification uncommon in untreated NSCLC. Next to NSCLC, *MET* has been found to be overexpressed in other cancer types as well such as gastric and esophageal cancer. *MET* overexpression results in an increase in cell proliferation and has anti-apoptotic properties. In addition, *MET* promotes cell-cell detachment, migration and invasiveness resulting in increased probability of developing distant metastases.

Expression of *MET* has also been shown to have a negative effect on NSCLC patient survival [88]. In patients who acquired resistance *MET* amplification is observed in 25% of all patients of which some of also had a second T790M mutation in EGFR [89]. Currently first trials of a c-MET inhibitor, crizotinib, are being performed to study its effect on tumor progression and could be used for a dual treatment with EGFR inhibitors to prevent the development of *MET* amplification [90].

Bevacizumab

In addition to cetuximab, bevacizumab is also a monoclonal antibody therapy which has been proven to increase survival in a number of different tumors. Clinical benefit of bevacizumab has been established in colorectal cancer and in metastatic breast and renal cancer [91, 92]. Bevacizumab binds to the vascular endothelial growth factor (VEGF) thereby sequestering the ligand for the VEGF receptor that plays a major role in angiogenesis. It has also been suggested that VEGF is a survival factor for tumor blood vessels making the tumor depend on the presence of VEGF. By inhibiting both neovascularization and affecting the integrity of tumor blood vessels, tumor growth and development can be targeted. It has been shown that inhibition of VEGF signaling indeed leads to the reduction of tumor vascularization and a decrease in tumor size [93]. Initial trials in advanced NSCLC patients, severe side effects were observed in squamous cell carcinoma patients. Hemorrhages were only observed in this subtype and were absent in other subtypes such as adenocarcinomas and large cell carcinoma. Wound healing however is impaired and a small increase in risk of serious bleeding was detected in all patients who were treated with bevacizumab. These side effects did not affect the survival gain of patients treated with the VEGF antibody [94]. Later trials excluded squamous cell carcinoma patients because of their

increased risk of hemorrhages and also in adenocarcinoma and large cell carcinoma populations had an increased survival time after treatment with bevacizumab. Reports have been made on a possible synergistic effect of combination therapy with cetuximab that increases the efficacy of both antibody regimens. However, trials performed using this combination have revealed a decrease in survival when both antibodies were administered at the same time [95-97]. Additional research is required to determine any beneficial effects of this combination treatment.

Future directions of non-small cell lung cancer treatment

Recently, additional agents have been successfully tested in NSCLC patients improving both survival and quality of life such as tyrosine kinase inhibitors and mono-clonal antibodies. Unfortunately, these targeted therapies have thus far only increased survival and not the cure rate due to new treatment regimens. Also, only a select group of patients are eligible to respond to these targeted therapies. Annual screening programs however have shown drastic increases in 5-year survival when tumors are identified in their 1a stadium. In combination with improved targeted therapy, personalized medicine and early detection, survival of NSCLC can improve significantly.

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