CHAPTER 1

Introduction

J.L. Kuiper
Chapter 1
LUNG CANCER

Incidence and risk factors
Lung cancer is the leading cause of cancer-related deaths worldwide (1). In 2015, in the Netherlands, more than 12,000 patients were diagnosed with lung cancer and almost 11,000 patients died of the disease (2). The lifetime risk of being diagnosed with cancer of the lung is approximately 6% (3). The most important risk factor for lung cancer is smoking; 85% of all newly diagnosed cases of lung cancer is associated with smoking (4). Yet, also in never-smokers, lung cancer ranks the seventh cause of cancer-related mortality worldwide (5). Geographical variations, exposure to occupational and domestic carcinogens, hormonal and environmental factors, as well as genetic predisposition may play a role as etiologic risk factors (6). The relatively high risk of developing lung cancer is probably also caused by stochastic effects associated with the lifetime number of stem cell divisions of lung tissue (7).

Classification of lung cancer
Lung cancer is histologically classified according to guidelines of the World Health Organization (WHO) (8) (Figure 1A). The predominant histological subtype of lung cancer is non-small cell lung cancer (NSCLC); approximately 80% of lung cancer patients is diagnosed with this type of lung cancer (9). The remaining part concerns small-cell lung cancer (SCLC) (14-18%) and other, more rare histological subtypes (i.e., mesothelioma and neuro-endocrine tumours) (6%) (2, 10). Among NSCLC, the predominating histological subtype is adenocarcinoma (approximately 40 – 45%) (11). Squamous cell carcinoma is diagnosed in 25 – 31% of NSCLC-patients and 9 – 18% of NSCLC is of the large-cell carcinoma subtype (10, 12).

Over the past decade, it has become evident that subsets of NSCLC can be further defined at the molecular level by a so-called ‘oncogenic driver’ (Figure 1B). These ‘driver’ mutations may occur in various oncogenes, including AKT1, ALK, BRAF, EGFR, HER2, KRAS, MEK1, MET, NRAS, PIK3CA, RET, and ROS1 (13). The cancer cell is mostly dependent on this particular mutated gene for its proliferation and survival. This phenomenon was described as the cancer cell being ‘oncogene addicted’ (14). This oncogenic deregulation can be caused by chromosomal translocations, gene amplifications or intragenic mutations, all eventually altering the function of the protein that the genes encode (15). ‘Driver’ mutations lead to constitutive activation of mutant signalling proteins that induce and sustain tumorigenesis.
Figure 1: Classification of lung cancer

Fig. 1A: Classification of lung cancer based on histology.
Fig. 1B: Classification of lung adenocarcinoma based on molecular features. The relative frequency of major driver mutations in signaling molecules in lung adenocarcinoma is shown.


Genetics in lung cancer

In lung cancer, the most prevalent mutations are found in the gene encoding the Kirsten rat sarcoma viral oncogene homolog (KRAS) - (29%), the epidermal growth factor receptor (EGFR) - (11%), V-Raf Murine Sarcoma Viral Oncogene Homolog B (BRAF) - (2%) and human epidermal growth factor receptor 2 (HER2) - gene (1%) (16). Rearrangements occur in the anaplastic lymphoma kinase (ALK) gene (5%) (16), Ret proto-oncogene (RET) gene (1%) (17) and C-Ros Oncogene 1 (ROS1) (1%) (18). Amplification has been described in HER2 (18%) (1, 19) and Hepatocyte Growth Factor Receptor MET (2-4%) (20). Nevertheless, there is a wide variance in incidence of these mutations between geographical regions worldwide. These oncogenic drivers occur predominantly in adenocarcinoma and existence is usually mutually exclusive.

Epidermal growth factor receptor

The protein EGFR is a member of the HER family, one of the 20 families of transmembrane receptor tyrosine kinases. Tyrosine kinases are enzymes that can attach phosphate groups to other amino acids. Phosphorylation of proteins by tyrosine kinases is an important mechanism for intracellular communication (signal transduction) and regulating cellular activity, such as cell division.

The HER family consists of the transmembrane receptors HER1 (ErbB1 or EGFR), HER2 (Neu, ErbB2), HER3 (ErbB3) and HER4 (ErbB4). Eleven growth factors have been described as ligand for EGFR, including epidermal growth factor (EGF), TGF-α, HB-EGF and amphiregulin (21). The tyrosine kinase part of EGFR is located intracellularly and is encoded by exons 18 - 24 (Figure 2). Upon ligand binding, intracellular signalling is triggered through the formation of hetero- or homodimers between HER-receptors. HER2 is the preferable partner of EGFR to dimerize after ligand binding. After formation of a hetero- or homodimer, the tyrosine residue is autophosphorylated. This autophosphorylation activates downstream signalling pathways including the MAPK, Akt and JNK pathways, ultimately leading to DNA-replication and cell division (22) (Figure 3). Under physiological circumstances, EGFR regulates cell processes like cell migration, adhesion and proliferation and has an important role in the innate immune response of the skin.
EGFR-mutations in cancer

Excessive signalling through the members of the HER-receptor family is associated with several types of cancer. Mutations in EGFR have been reported in a variety of cancers, including anal cancer, glioblastoma multiforme and lung cancer (23, 24). In lung cancer, 90% of all EGFR-mutations are represented by mutations in two regions, often referred to as the ‘classic’ activating EGFR-mutations (25). These mutations concern in-frame deletions in exon 19 (usually around the amino acids Glycine-Leucine-Arginine-Glycine-Alanine (ELREA), residues 746-750) (45 – 50%) and the Leu858Arg (L858R) substitution, resulting from a point mutation in exon 21 (40 – 45%) (26). Classic EGFR-mutations increase the kinase activity of the protein, thereby continuously activating the downstream pro-survival pathways in absence of ligand-binding (27). The remaining 10% of EGFR-mutations concern so-called ‘non-classic mutations’ (or: ‘uncommon’ mutations) in exons 18 – 21.

EGFR-mutations are reported to occur almost exclusively in non-squamous NSCLC (8). 9.4% of Caucasian NSCLC-patients and up to 47.9% of Asian NSCLC-patients carry EGFR-mutations in their tumours (16, 28). Regardless of ethnicity and histology, clinical characteristics that are associated with EGFR-mutations are non-smoking (14 – 56%) and female gender (20 – 62%) (26, 29-32), however EGFR-mutations are also reported in males (1 – 19%) and smokers (3 – 14%) as well (26).

Stage, treatment options, survival and prognosis of NSCLC

Whether curative treatment options are available for a patient with NSCLC is largely dependent on the stage of disease at time of diagnosis. After pathological confirmation of the presence of lung cancer, the characteristics of the primary tumour (T), the involvement of regional lymph nodes (N) and the presence or absence of distant metastasis (M) are evaluated in order to establish a TNM-classification. From this TNM stage, the stage of disease (stage I – IV) can be determined (4, 33).
Patients with local (stage IA – IIB) and locally advanced NSCLC (IIIA) can be curatively treated with surgery, (stereotactic) radiotherapy and/or chemotherapy. However, due to a lack of symptoms in the early stages of disease, the vast majority of NSCLC-patients (more than 80%) have advanced-stage disease (stage IIIB – IV) at time of first diagnosis. For advanced-stage NSCLC patients there are currently no curative treatment options available (4). Nonetheless, (combinations of) new treatment modalities have been investigated in recent years and have improved treatment outcomes of subsets of advanced-stage NSCLC.

Prognosis of advanced-stage lung cancer is to some extent dependent on biological and clinical factors, such as weight loss, histological and molecular subtype, and performance status (PS) (34). Despite improvement in the treatment of advanced-stage NSCLC, overall survival (OS) is still poor. Median OS after diagnosis of stage IV NSCLC adenocarcinoma is 12.6 months (35) and the five-year survival rate of unselected patients with stage IV NSCLC is less than 5% (1). EGFR-mutated NSCLC-patients have a better prognosis compared to the unselected NSCLC-population; for these patients a five-year survival rate of 14% has been reported (36).

Figure 3: EGFR signalling pathways.
**Treatment of advanced stage lung cancer**

Since the discovery of platinum-based chemotherapeutic agents as anti-cancer treatment in the 1970s, these drugs have been the standard first-line treatment for advanced-stage lung cancer (37). Until the 1990s all advanced-stage lung cancer patients were treated with a platinum-based chemotherapeutic regimen (usually combined with etoposide). From 2000, the distinction between SCLC and NSCLC became clinically relevant, when it was demonstrated that specific chemotherapeutic regimens have different efficacy in SCLC and NSCLC (38).

First-line treatment with chemotherapy improves overall survival of stage IV NSCLC-patients by 9% (39). The optimal duration of first-line platinum-based chemotherapy is considered to be four cycles (39, 40). A two-agent regimen of cytotoxic chemotherapy provides the optimal effect considering tumour response and overall survival compared to one- or three-agent regimens (41). The histological subtype of NSCLC may guide the selection of the second chemotherapeutic agent, next to the platinum-based chemotherapeutic agent. Patients with squamous cell carcinoma should not be treated with the third-generation chemotherapeutic agent pemetrexed, but receive a different agent (e.g. gemcitabine) (42).

The last couple of decades it is increasingly recognized that targeted small molecule inhibitors can be worthwhile treatment modalities for specific molecularly defined subsets of lung cancer patients, such as those with an *EGFR*-mutation, *ALK*-rearrangement and possibly other drugable activating mutations. For this reason, all patients with stage IV adenocarcinoma of the lung in the Netherlands should routinely be tested for at least *EGFR*-mutations and *ALK*-rearrangements to guide treatment decisions (4).
TARGETED TREATMENT OF EGFR-MUTATED NSCLC-PATIENTS

EGFR-inhibition in lung cancer
EGFR-tyrosine kinase inhibitors (TKIs) are small molecule TKIs that bind to the tyrosine kinase domain of the EGFR. The catalytic activity of the tyrosine kinase is blocked by the competition of these agents with adenosine triphosphate (ATP). Gefitinib (ZD1839, Iressa) and erlotinib (OSI-774, Tarceva) bind reversibly to the EGFR and were the first EGFR-TKIs to be registered for lung cancer treatment. The EGFR-TKI afatinib (BIBW2992, Giotrif) is a pan-HER binding agent and was the subsequent EGFR-TKI to be registered for the treatment of lung cancer.

In 2003, the Food and Drug Administration (FDA) granted accelerated proof for gefitinib after evaluation in previously treated NSCLC-patients in two randomized phase II trials (43, 44). Performance status (PS), histology and female gender were clinical parameters that were associated with a response. Most frequently reported toxicities were acne-like rash and gastro-intestinal side effects. The second TKI that received FDA-approval for treatment of patients with advanced stage NSCLC was erlotinib, after evaluation in the randomized, placebo-controlled, double-blind BR.21 trial (45). Retrospective subset analyses showed that also adenocarcinoma and smoking status were associated with response to erlotinib.

A phase III trial evaluated gefitinib in previously treated NSCLC-patients (46), but no difference in survival was detected. However, in this trial, it was suggested that EGFR-mutations might be the predictive biomarker for response to EGFR-TKIs (47). The INTEREST trial was the first phase III trial that demonstrated that NSCLC-patients with EGFR-mutations had longer PFS and higher ORR when treated with gefitinib compared to docetaxel (48).

The discovery that EGFR-mutations were the biomarker for prediction of response to EGFR-TKI treatment in 2004 (29, 30), led to the initiation of a variety of phase III randomized trials evaluating EGFR-TKIs in EGFR-mutated NSCLC patients (Table 1). All of these trials demonstrated the beneficial effect of EGFR-TKIs as compared to cytotoxic chemotherapy in NSCLC-patients carrying an EGFR-mutation, in terms of prolongation of PFS and improved response rate. Since then, first-generation EGFR-TKIs are incorporated as first-line treatment for EGFR-mutated NSCLC-patients in international guidelines (49-51). Erlotinib is also registered for second-line treatment of unselected NSCLC-patients, based on the results of the BR.21 trial (45). Despite the evident effect of erlotinib and gefitinib on PFS and response rates, a beneficial effect on overall survival has never been demonstrated which is probably due to cross-over of patients to EGFR-TKIs after treatment with chemotherapy (52).

Unfortunately, resistance to EGFR-TKIs inevitably develops after a median of 8.0 – 13.1 months in TKI-naive patients (53-60). It was hoped that the irreversible second-generation EGFR-TKI afatinib could overcome this resistance. Afatinib had been demonstrated to be highly effective in preclinical lung cancer models, including erlotinib-resistant isoforms (61). Afatinib obtained FDA-approval in 2013 for treatment-naive NSCLC-patients with an EGFR-mutation.
First-line afatinib improved OS in two phase III trials for patients with a exon 19 deletion, but not for patients with an L858R point mutation (62). However, the results of afatinib in NSCLC-patients with acquired resistance to erlotinib or gefitinib were disappointing; response rate was only 8.2% (63) and OS was not prolonged in a placebo-controlled study (64).

**Resistance mechanisms in EGFR-TKI treatment**

Mechanisms of acquired EGFR-TKI resistance can be grouped into three different categories: EGFR modification, alternative pathway activation and histologic transformation (65). The most prevalent mechanism of resistance in EGFR-mutated NSCLC-patients after TKI-treatment is the secondary T790M mutation, which occurs in 50 – 60% of EGFR-mutated NSCLC-patients with acquired resistance to first-generation EGFR-TKIs (66, 67). This mutation is located in exon 20 of EGFR and abrogates the binding of reversible EGFR-TKIs with the ATP-binding pocket of the EGFR kinase domain by increasing the affinity of the receptor for ATP by approximately five-fold (68-70). Amplification of alternative pathways has also been described as a mechanism of resistance, e.g. MET-amplification or HER2-amplification (71-73). Due to activation of by-pass tracks, downstream signalling can be maintained, despite inhibition of EGFR. Histologic transformation is detected in a minority of patients with acquired TKI-resistance, e.g. transformation from NSCLC to SCLC is detected in 3% of the patients with acquired EGFR-TKI resistance (67).
Table 1 Phase III trials evaluating EGFR-TKIs as 1st-line treatment in EGFR-mutated NSCLC-patients

<table>
<thead>
<tr>
<th>Author (et al)</th>
<th>Trial</th>
<th>Journal, year</th>
<th>TKI</th>
<th>PFS (months)</th>
<th>ORR</th>
<th>OS (months)</th>
<th>Ethnicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maemondo (54)</td>
<td>NEJ002</td>
<td>NEJM, 2010</td>
<td>Gefitinib vs. CT</td>
<td>10.8 vs. 5.4</td>
<td>73.7% vs. 30.7%</td>
<td>30.5 vs. 23.6</td>
<td>Asian</td>
</tr>
<tr>
<td>Mitsudomi (74)</td>
<td>WJOG3405</td>
<td>Lancet Oncol 2010</td>
<td>Gefitinib vs. CT</td>
<td>9.2 vs. 6.3</td>
<td>62.1% vs. 32.2%</td>
<td>Immature Asian</td>
<td></td>
</tr>
<tr>
<td>Rosell (56)</td>
<td>EURTAC</td>
<td>Lancet Oncol 2012</td>
<td>Erlotinib vs. CT</td>
<td>9.7 vs. 5.2</td>
<td>63.6% vs. 17.8%</td>
<td>19.3 vs. 19.5 Caucasian</td>
<td></td>
</tr>
<tr>
<td>Zhou (58)</td>
<td>OPTIMAL</td>
<td>Lancet Oncol 2011</td>
<td>Erlotinib vs. CT</td>
<td>13.1 vs. 4.6</td>
<td>83% vs. 36%</td>
<td>Immature Asian</td>
<td></td>
</tr>
<tr>
<td>Mok (75) and</td>
<td>IPASS</td>
<td>J Clin Oncol 2011</td>
<td>Gefitinib vs. CT</td>
<td>NA</td>
<td>84.8% vs. 43.2%</td>
<td>21.6 vs. 21.9 Asian</td>
<td></td>
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<tr>
<td>Fukuoka (43)</td>
<td></td>
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<tr>
<td>Han (53)</td>
<td>First-Signal</td>
<td>J Clin Oncol 2012</td>
<td>Gefitinib vs. CT</td>
<td>8.0 vs. 6.3</td>
<td>84.6% vs. 37.5%</td>
<td>27.2 vs. 25.6 Asian</td>
<td></td>
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<tr>
<td>Sequist (57)</td>
<td>Lux-lung 3</td>
<td>J Clin Oncol 2013</td>
<td>Afatinib vs. CT</td>
<td>11.1 vs. 6.9</td>
<td>56% vs. 23%</td>
<td>16.6 vs. 14.8 Predominantly Asian</td>
<td></td>
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<tr>
<td>Wu (60)</td>
<td>Lux-lung 6</td>
<td>Lancet Oncol 2014</td>
<td>Afatinib vs. CT</td>
<td>11.0 vs. 5.6</td>
<td>66.9% vs. 23.0%</td>
<td>22.1 vs. 22.2 Asian</td>
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Legends: CT: chemotherapy, vs: versus
OUTLINE OF THIS THESIS

Lung cancer is the leading cause of cancer related mortality in the Netherlands, indicating that novel therapies and improved treatment strategies are urgently needed. Classically, treatment decisions have been empiric and mainly based upon histology of the tumour. Over the past decade, it has become evident that subsets of NSCLC can be further defined at the molecular level by recurrent ‘driver’ mutations. One of such altered genes is EGFR, which is mutated in approximately 10% of NSCLC-patients in the Netherlands (76). Importantly, targeted small molecule inhibitors are currently available for EGFR-mutated lung cancer patients and have become the standard of treatment for these patients. The introduction of these first-generation EGFR-TKIs in the treatment of lung cancer has been a major step forward, yet new challenges and questions have arisen as well. This thesis describes the results of clinical studies conducted among EGFR-mutated NSCLC-patients and NSCLC-patients with acquired resistance to first-generation EGFR-TKIs. The first part focuses on diagnostics and predictive markers, and the second part focuses on treatment.

**Diagnostics & response prediction**

*Prevalence of uncommon EGFR-mutations*

Studies that report on non-classic EGFR-mutations are limited and data on EGFR-TKI sensitivity of these mutations is scarce, especially in non-Asian populations. In Chapter 2, we describe the prevalence and type distribution of non-classic EGFR-mutations among Dutch EGFR-mutated NSCLC-patients, as well as clinical characteristics and outcomes on EGFR-TKI treatment in this cohort.

*T790M mutation as mechanism of EGFR-TKI-resistance*

The T790M mutation is the most frequently detected mechanism of resistance in EGFR-mutated NSCLC-patients after having acquired resistance to EGFR-TKI treatment. The mutation is rarely found independently of EGFR-TKI treatment, however some patients in whom the T790M mutation was detected prior to EGFR-TKI treatment have been reported (77). Heterogeneity of T790M detection has been described in individual cases, both in time (78) and between different tumour lesions (79). In Chapter 3, we describe the occurrence of T790M mutations in a cohort of EGFR-mutated NSCLC patients who were rebiopsied after having acquired EGFR-TKI-resistance.

*Histological transformation and tumour heterogeneity*

The existence of several populations of cancer cells with different morphological and/or molecular characteristics in patients is called tumour heterogeneity (80). When targeted agents were introduced in the treatment of cancer, the role of genetic tumour heterogeneity
has gained interest given its likely role in the development of resistance to treatment. In Chapter 4a, we describe an EGFR-mutated NSCLC-patient in whom an innovative PET imaging method was used that could possibly play a role in detecting tumour heterogeneity in the future.

Histological transformation after treatment with EGFR-TKIs, e.g. transformation from NSCLC to SCLC, has been described as resistance mechanism, but is infrequently reported (67). Histological transformation may develop as a result of tumour heterogeneity that was present prior to treatment initiation. In Chapter 4b we describe a patient who was rebiopsied after acquired resistance to EGFR-TKI treatment and in whom a different form of histologic transformation after EGFR-TKI treatment was reported; transition of an adenocarcinoma to squamous cell carcinoma phenotype.

**Serum-based proteomic biomarkers**

Although the presence of an EGFR-mutation in the tumour is predictive for response on EGFR-TKI treatment, some EGFR-wild type (WT) patients experience prolongation of PFS when treated with EGFR-TKI treatment (45). Moreover, in some patients assessment of tumour mutation-status is either not possible or it is not possible to obtain (new) tumour-tissue. Therefore, it would be helpful to have a less-invasive biomarker test available that is predictive for response to EGFR-TKI treatment.

VeriStrat is a serum-based proteomic test that is commercially available in the United States and has shown to predict outcome after treatment with EGFR-TKIs (81-85). Chapter 5 describes the results of the VeriStrat-test in a cohort of unselected NSCLC-patients who were treated with the combination of erlotinib and sorafenib.

**Treatment**

*Present-day challenges in EGFR-TKI-resistant NSCLC*

Since two decennia, response to anti-cancer treatment is measured according to the response evaluation criteria in solid tumors (RECIST) (86). One of the important issues is whether traditional response evaluation criteria are still applicable in the treatment with targeted agents. This issue is reviewed in Chapter 6, as well as the mechanisms of resistance and different treatment strategies in EGFR-mutated NSCLC with acquired EGFR-TKI resistance.

*Leptomeningeal metastases*

A substantial part of NSCLC-patients develop brain metastases (87). It is hypothesized that EGFR-mutated NSCLC-patients have a higher chance of developing metastases in the brain, because of their prolonged survival compared to EGFR-WT NSCLC-patients (88). A separate entity among brain metastases are leptomeningeal metastases (meningitis carcinomatosis). Leptomeningeal metastases occur when malignant cells enter the subarachnoid space within
the compartment of the cerebrospinal fluid (89). It is considered to be a complication with poor prognosis and rapid deterioration of performance status (90). In Chapter 7, we describe diagnosis, treatment and survival of EGFR-mutated NSCLC-patients with leptomeningeal metastases.

**Pulsatile EGFR-TKI treatment**

It is believed that, due to the blood-brain barrier (BBB), EGFR-TKIs do not reach therapeutic concentrations in the intra-central nervous system (CNS) compartment when administered in standard dose (91). It is hypothesized that with high-dose weekly erlotinib, therapeutic concentrations in the intra-CNS fluid can be reached (92). In Chapter 8a, we describe two EGFR-mutated NSCLC-patients who developed leptomeningeal metastases and both responded to high-dose EGFR-TKI treatment. Interestingly, one patient also had a response of the intrathoracic lesions that had been resistant for previous chemotherapy.

Since EGFR-TKIs are competitive inhibitors of EGF signalling, it can be hypothesized that higher doses of the drug might restore the sensitivity. The results of a phase II trial that evaluated high-dose weekly EGFR-TKI treatment in EGFR-mutated NSCLC-patients who acquired resistance to standard-dose EGFR-TKI treatment, are described in Chapter 8b.

**Afatinib**

In pre-clinical studies, erlotinib-resistant cell lines were sensitive for treatment with afatinib (61). Subsequently, this agent was evaluated in NSCLC-patients with acquired resistance to erlotinib or gefitinib (63, 64). However, in these studies there was no molecular restriction for patient selection. We therefore retrospectively evaluated a cohort of NSCLC-patients with acquired resistance to erlotinib and/or gefitinib who were rebiopsied prior to treatment with afatinib. Preliminary results of this study are described in Chapter 9.
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