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Primary and secondary resistance mechanisms in EGFR mutated non-small cell lung cancer

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General introduction and outline of this thesis

General aspects of lung cancer

In 2020, lung cancer remained the leading cause of cancer death worldwide with an estimated 1.80 million deaths (1). Also in the Netherlands, lung cancer has a high mortality rate; although survival rates are still improving, only 21% of the patients are alive 5 years after diagnosis (based on the period 2011 – 2018) (2). In 2019, more than 14.000 people were diagnosed with lung cancer in the Netherlands. Approximately 50% of them has stage IV disease at diagnosis, this means the cancer has spread to distant organs and is in general incurable (2). Lung cancer is mostly a disease of the elderly, around 70% of patients with stage IV disease is 65 years or older (2).

Lung cancer can be divided into two subgroups, non-small cell lung cancer (NSCLC) and small cell lung cancer. Eighty five % of lung cancer patients are classified as NSCLC, a collective name for different subtypes: including adenocarcinoma, squamous cell carcinoma and other less common subtypes. Most lung carcinomas are adenocarcinomas.

Tobacco smoking remains the leading cause of lung cancer (3). The number of lung cancer patients who have never smoked varies widely, from more than 50% in women in Southeast Asia, to approximately 2-6% in men in Western countries (4). Lung cancers that occur in never smokers, defined as less than 100 cigarettes over lifetime, are considered a distinct entity (4, 5). Causative factors in this population are still poorly understood. The demographics of non-smokers with lung cancer are different compared with smokers, with more women and individuals of younger age. The vast majority are adenocarcinomas. In addition, never-smoker lung cancers show a high prevalence of targetable oncogenic driver alterations in nearly 80% of patients of whom approximately 50% harbors an epidermal growth factor receptor (*EGFR*) mutation (6, 7).

The majority of NSCLC patients are diagnosed with advanced disease on first presentation and are unsuitable for curative treatment. Until 2004, the standard treatment for advanced stage NSCLC was platinum based chemotherapy, with modest effects on life expectancy (8).

Over the past two decades, the therapeutic landscape of lung cancer has changed tremendously. The discovery of small-molecule receptor tyrosine kinase inhibitors (TKIs) was regarded as a landmark finding and has brought impressive clinical activity in specific subgroups with oncogenic drivers activated by mutation, translocation or fusion. Molecular analyses to detect oncogenic drivers have become standard in patients with advanced stage disease. The Dutch guideline on NSCLC recommends to test every patient with stage IV adenocarcinoma or never-smokers, regardless their histology, for the following driver alterations: *KRAS*, *EGFR*, *BRAF*, *HER2*, *ALK*, *MET*, *ROS1*, *RET*, *NTRK1-3* and *NRG1* (9). In the Netherlands, 50% of patients harbors a molecular alteration, mostly *KRAS* (37.9%), followed by *EGFR* (10.5%) and *BRAF* (4.1%) (10). Unfortunately, only a minority of lung cancer patients harbor a targetable genetic alteration; therefore, most NSCLC patients are not eligible for targeted treatment. This thesis focusses on patients with metastatic NSCLC harboring an *EGFR* mutation.

EGFR mutations

The EGF receptor is a member of the ErbB family, which consists of four related receptor tyrosine kinases: EGFR or HER1 (ErbB-1), HER2neu (ErbB-2), HER3 (ErbB-3) and

HER4 (ErbB-4). It is a transmembrane protein receptor activated by binding its specific ligands, including EGF and transforming growth factor- α (11). Binding of EGFR to its ligands results in receptor dimerization followed by autophosphorylation, which leads to downstream activation of the Ras-Raf mitogen-activated protein kinase and the phosphatidylinositol 3' kinase and Akt pathways. These pathways regulate cell proliferation, angiogenesis and inhibition of apoptosis (12). The physiological activity of the EGF receptor is tightly regulated, otherwise it can lead to uncontrolled cell growth and cancer formation (13).

The epidermal growth factor receptor is frequently overexpressed in common solid tumors and in 50% of NSCLCs (13). This observation made EGFR an interesting target for the development of targeted agents. In May 2003, the EGFR inhibitor gefitinib was approved as third-line therapy for non-small cell lung cancer (14, 15). In general, response rates were low in approximately 10% of patients. However, a small subset of patients showed dramatic, long-lasting responses, independent of EGFR overexpression levels (16, 17). Thereafter, Lynch et al. sequenced the entire coding region of EGFR in tumour samples from patients with a response and found different somatic gain-of-function mutations within the tyrosine kinase domain of EGFR (18). This was the starting point of the discovery of targeted therapy in lung cancer.

The *EGFR* gene is located on chromosome 7 short arm q22 and consists of four domains: an extracellular domain (EGF binding), transmembrane domain and two intracellular domains, the tyrosine kinase and autophosphorylation regions (19). The tyrosine kinase domain can be divided into an N-lobe and a C-lobe, with an ATP-binding site located between the two lobes (19). *EGFR* mutations in NSCLC are almost exclusively clustered within the tyrosine kinase domain of EGFR, and more specifically between exon 18 and 21, around the ATP-binding pocket.

EGFR in-frame deletions in exon 19 are the most prevalent *EGFR* mutations, accounting for 45% of all *EGFR* mutations, followed by *EGFR* L858R missense mutation in exon 21, which is found in 40% of *EGFR* kinase domain mutations. Exon 20 insertions (ex20ins) comprise 4-12% and are the third most common category of *EGFR* mutations found in NSCLC. The remaining *EGFR* mutations consist of a small group of nucleotide substitutions in exon 18 and 20 (13, 20, 21).

Classic *EGFR* mutations

Approximately 85% of *EGFR* mutations comprise the two classic activating *EGFR* mutations: in-frame deletions in exon 19 and L858R substitution mutations in exon 21. Activation of wild-type EGFR is primarily driven by ligand-binding-induced dimerization. Classic *EGFR* mutations hyperactivate the kinase activity of EGFR by stabilizing ligand-independent dimerization with ERBB family receptors (19). Of all *EGFR* mutations, *EGFR* exon 19 deletions exhibit the highest catalytic activity, followed by L858R mutations (22-24). These mutations increase the relative affinity for EGFR TKIs over ATP compared with wild-type EGFR.

Treatment

Two classes of EGFR directed therapies have been developed since 1984. First anti-EGFR monoclonal antibodies (mAb), and in 2003 early generation reversible EGFR TKIs were introduced (25, 26).

The role of anti-EGFR mAb's in the treatment of *EGFR*-mutated NSCLC is limited as they are ineffective as a single agent (27). Anti-EGFR mAb's bind to the extracellular domain of EGFR and block ligand-induced EGFR tyrosine kinase activation by occluding the ligand-binding region (25, 26). Cetuximab was the first anti-EGFR human-to-murine chimeric mAb tested in patients with EGFR overexpression determined by immunohistochemical analysis. Treatment with cetuximab alone was well tolerated. The most frequent adverse events were fever and chills, asthenia, transaminase elevation, nausea, skin toxicities (flushing, dermatitis and acneiform rashes) and allergic reactions (28).

Small-molecule EGFR TKIs like gefitinib and erlotinib, compete reversibly with ATP to bind to the receptor's kinase pocket, which inhibit EGFR autophosphorylation and prevents downstream signaling (26, 29). Gefitinib and erlotinib, two first-generation EGFR TKIs, have shown an improvement of objective response rate (ORR) and progression free survival (PFS) compared to platinum-based chemotherapy and became the standard of care as first-line treatment for advanced *EGFR* mutated NSCLC (30-32). Dose-dependent and reversible diarrhea and acneiform rashes are the most frequent side effects. Thereafter, irreversible second-generation EGFR TKIs (afatinib and dacomitinib) did show a slight clinical improvement compared to first-generation TKIs, however they were associated with higher toxicity rates (33-35). Until the discovery of third generation EGFR TKIs, first- or second generation EGFR TKIs were both used without a clear preference.

Despite significant clinical and durable benefit after treatment with early-generation EGFR TKI's, patients inevitably develop disease progression, mostly driven by acquisition of an exon 20 T790M resistance mutation. This mutation, also referred to as 'gatekeeper' mutation, sterically hinders the binding of early-generation EGFR TKIs to the ATP-binding site of EGFR. Osimertinib, a third generation EGFR TKI, was developed to selectively inhibit both EGFR-TKI-sensitizing and *EGFR* exon 20 T790M resistance mutations while sparing wild-type EGFR (36). As first-line treatment, it also demonstrated a significantly PFS and overall survival (OS) benefit relative to first-generation EGFR TKIs. In addition, osimertinib showed superior activity against brain metastases and has a favorable toxicity profile (37-40). With a median overall survival of more than three years, first-line osimertinib is the preferred option for *EGFR*-positive NSCLC patients at this time (41).

Immune checkpoint inhibitors has revolutionized the treatment of NSCLC, however the efficacy of immunotherapy in *EGFR*-mutated NSCLC appears to be quite limited (42). In addition, the use of immune checkpoint inhibitors prior to or concurrent with osimertinib increases the risk of pneumonitis (43). This underlines the importance of molecular testing at diagnosis to enable personalized treatment.

***EGFR* exon 20 insertion mutations**

EGFR ex20ins mutations comprise approximately 1 to 2% of all NSCLC cases and 4 – 12% of all *EGFR* mutated lung cancers, representing the third most common type of *EGFR* mutations (20, 21, 44, 45).

Similar to the classic *EGFR* mutations, *EGFR* ex20ins are more common among never-smokers and females. The increased incidence among Asian patients is less pronounced. In addition, the ex20ins mutation was more common in older patients compared to *EGFR* exon 19 deletions and L858R mutations (20, 46). The prognosis of patients harboring an *EGFR* ex20ins mutation is worse, compared to patients with common or other uncommon *EGFR* mutations. The median OS ranged from 4.8 – 20 months, similar or slightly better than the *EGFR* wild-type population (46-48).

EGFR ex20ins mutations are a heterogeneous group of activating mutations within the tyrosine kinase domain of EGFR. They represent a combination of in-frame insertions and/or duplications, ranging from three to 21 base pairs, clustered between amino acid positions 762 and 774 of exon 20 (20). This region of exon 20 contains two important regions: the regulatory C-helix (762 – 766) and the adjacent loop that follows it (767-774) (20, 21, 45, 49). To date, more than 60 unique variants are identified, mostly located in the loop following the C-helix (45).

PD-L1 status is negative in approximately 75% of patients with an *EGFR* ex20ins mutation and median tumor mutational burden is low (48, 50). Although little data is available, they seem to derive less benefit from immunotherapy (50).

Treatment

EGFR ex20ins are generally associated with de-novo resistance to early generation EGFR TKIs, except for variant A763_Y764insFQEA (20, 46, 51, 52). Classic *EGFR* mutations directly affect the structure of the ATP-binding pocket and increase the relative affinity for EGFR TKIs over ATP compared with wild-type EGFR. This leads to a large therapeutic window for EGFR TKIs. Targeting *EGFR* ex20ins is challenging because they activate EGFR without diminishing ATP affinity, which makes it difficult to selectively target the ex20ins mutant over wild-type EGFR. In addition, *EGFR* ex20ins mutations induce steric hindrance of the drug-binding pocket by pushing the C-helix into its inward (active state) position, which prevents binding of EGFR TKIs (53). Insertions before residue 764, like the A763_Y764insFQEA variant, do not reveal this stabilized active formation and do not induce drug resistance (21). The role of osimertinib had not yet been established. 3D modeling of the crystal structures of *EGFR* ex20ins showed changes within the drug-binding pocket, similar to T790M (53). Given these similarities, osimertinib could be a treatment option. However, in contrast with T790M mutation, *EGFR* ex20ins mutations are characterized by changes of the a-C helix in an inward, activated position. In addition, 3D modeling showed a shift of the P-loop into the drug-binding pocket, resulting in steric hindrance of the drug-binding pocket from different directions (53). Osimertinib has a large inflexible terminal 1-methylindole group that reduces the binding ability to *EGFR* ex20ins. So based on preclinical data, the role of osimertinib for this patient category was unclear. Overall, platinum-based chemotherapy is to date the most efficacious first-line treatment for patients with an *EGFR* ex20ins mutation (48, 54).

Outline of this thesis

The primary goals of this thesis were to gain insights in primary and secondary resistance mechanisms in patients with *EGFR* mutated NSCLC and to develop new treatment strategies.

The first part of this thesis focuses on a specific subgroup of *EGFR* mutated lung cancer patients, namely patients harboring an *EGFR* ex20ins mutation. We retrospectively and prospectively analyzed the activity of two different treatment regimens for this specific mutation subtype.

The second part focusses on genomic profiling of resistance mechanisms after treatment with a third generation EGFR TKI. In addition, we investigated the activity of combination treatment after cMET driven resistance.

PART I. Targeted treatment of *EGFR* exon 20 insertion mutations

This part of the thesis focuses on finding new targeted treatment options for patients with advanced stage NSCLC harboring an *EGFR* ex20ins mutation. Although the clinical activity of osimertinib for classical *EGFR* mutations is impressive, data about effectiveness of third generation *EGFR* TKIs for patients harboring an *EGFR* ex20ins mutation were conflicting in pre-clinical studies. Limited clinical data were available. Firstly, we retrospectively collected clinical and molecular data of 21 patients harboring an *EGFR* ex20ins mutation who were treated with osimertinib to gain insight into effectiveness. In [chapter 2](#), we described the results of this case series. Almost all patients were treated with osimertinib 80 mg once daily with limited efficacy. Preclinical data showed that the IC50 values of osimertinib for *EGFR* ex20ins mutations were 10 to 100 fold higher compared to classical *EGFR* mutations. We hypothesized that a higher dose of osimertinib might be necessary to effectively treat those patients. In [chapter 3](#), we present the results of the POSITION-20 trial, a prospective trial in which 25 patients harboring an *EGFR* exon 20 mutation were treated with high-dose osimertinib in first or second line.

A different strategy to target *EGFR* ex20ins positive NSCLC could be to intensify *EGFR* blockade by concurrent TKI and *EGFR* monoclonal antibody treatment. Afatinib is an interesting drug to combine with other *EGFR* directed therapies like anti-*EGFR* mAbs, given its broad and irreversible inhibitory profile and low potential for drug interactions (55). First-line afatinib plus cetuximab already showed efficacy in heavily pretreated *EGFR* -mutant NSCLC patients with acquired resistance to first generation *EGFR* TKIs (56). Dual *EGFR* blockade with afatinib and cetuximab may also induce tumor responses in *EGFR* ex20ins mutations, based on the treatment outcomes of four *EGFR* exon 20 insertion positive NSCLC patients treated with this combination, presented in [chapter 4](#). We tried to validate these results in a dedicated clinical trial. In [chapter 5](#), we present the results of a single-arm, phase II trial, where 37 advanced *EGFR* ex20ins positive treatment-naïve or pretreated NSCLC patients were treated with afatinib in combination with cetuximab.

In recent years, several TKIs directed against *EGFR* ex20ins mutations have been developed. However, *EGFR* ex20ins mutated NSCLC cannot be seen as a single disease entity, due to marked heterogeneity in this subgroup. There is a need to expand the knowledge on the structure-function relationship of different *EGFR* ex20ins mutants on drug sensitivity. [Chapter 6](#) provides a comprehensive assessment of the available literature of pre-clinical and clinical drug sensitivity to currently available TKIs across different *EGFR* ex20ins mutations.

PART II. Analysis an atment of resistance mechanisms after third generation *EGFR* TKI treatment.

Despite robust activity of osimertinib in first- and second-line settings, eventually acquired resistance occurs. In [chapter 7](#), we retrospectively analyzed pre- and post-treatment biopsies in patients who received a third-generation *EGFR* TKI in second line to gain more insights in innate and acquired resistance mechanisms. Bypass-track mechanisms after third-generation *EGFR* TKI treatment are most commonly MET amplifications. Little was known about the efficacy of cMET inhibitors in the acquired resistance setting. Finally, in [chapter 8](#) we retrospectively analyzed the treatment results of patients with acquired cMET amplification after *EGFR*-directed therapy, who are treated with crizotinib.

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