FUTURE TREATMENT OF NSCLC PATIENTS WITH NEW EGFR TARGETING DRUGS

Avoidance of EGFR-TKI resistance through inhibition of C-MET and the use of new generation EGFR-TKI’s

Mutated EGFR kinase complex in association with gefitinib (Michalczyk et al. 2008).

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ABSTRACT
The treatment of non-small cell lung carcinoma (NSCLC) patients with epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI’s), such as gefitinib and erlotinib, was proven to be effective in a subset of patients with a mutated constitutive active EGFR. However, during treatment the cancer cells evolve and develop resistance against these EGFR-TKI’s, causing a major hurdle for successful treatment. A number of mechanisms were identified responsible for this type of EGFR-TKI resistance; the most common are the gatekeeper EGFR-T790M mutation and C-Met amplification. Some patients have NSCLC due to a ROS1 or anaplastic lymphoma kinase (ALK) chromosomal rearrangement while they have the same pathology. The most efficient methods for this identification are fluorescent in situ hybridization’ (FISH) and immuno- histochemistry (IHC). To overcome resistance, new drugs like crizotinib were used to target the C-Met receptor. Furthermore new generation EGFR-TKI’s including afatinib and WZ4002, have been developed. The efficacy of these new drugs was tested in different type of NSCLC cells, in various in vitro and in vivo models. The combination of WZ4002 with crizotinib showed the most dramatic effect against the mutated cells, while the side effects and toxicity were marginal compared to the afatinib and crizotinib combination. Thus, the use of crizotinib and WZ4002 can overcome EGFR-TKI resistance the case of a mutated EGFR.

INTRODUCTION
Lung cancer is the most lethal form of cancer throughout the world, 28% of the male and 26% of all woman cancer deaths are due to lung cancer (Siegel et al. 2014). 85% of these patients have NSCLC. Often these patients are non-smokers, while other types of lung cancer like small cell lung carcinoma (SCLC) are usually more correlated with smoking (Selinger et al. 2013). NSCLC is a large group of epithelial lung cancer types like large cell carcinoma, adenocarcinoma and squamous cell carcinoma. Histologically these cancer types have larger cells compared to SCLC. Unfortunately, NSCLC is quite insensitive to chemotherapy and this type of cancer easily metastasizes throughout the body. Patients without a metastasis are usually treated with surgery and radiation. Although NSCLC is insensitive for chemo-therapy the old fashion way to treat this type of cancer was with conventional cytotoxic chemicals, especially if the tumor metastasizes. These chemicals induce DNA damage, inhibit DNA synthesis or the uptake of nutrients and therefore proliferation. Since these processes are obviously essential to all dividing cells in the body, these drugs generally cause severe side effects (Ciarimboli 2014). Even if this approach is combined with surgery and radiation, it is in most cases not enough to eradicate the disease (Zhang et al. 2007). This is why there is a lot of research nowadays to identify and develop alternative drugs to effectively treat NSCLC patients. Because the tumor is often depending on specific pathways that drive proliferation, these drugs are targeted on specific components of those pathways. The RAS and Akt pathways for instance, which can be activated with the EGFR, can lead to NSCLC pathology (Figure 1). In the case of EGFR related NSCLC a new approach is used to effectively inhibit the receptor with either antibodies or tyrosine kinase inhibitors (TKI), this essay will primarily focus on the latter one. One of those drugs are first generation EGFR-TKI’s like gefitinib and erlotinib. Although these drugs work quite well against NSCLC, resistance is becoming a major problem. So the main question of this thesis is: What is the cause of EGFR-TKI resistance and how can it be prevented?

NSCLC IN RELATION WITH EGFR
About 15% of the Caucasian and 30-60% of the Asian NSCLC patients have a mutation in the EGFR (Selinger et al. 2013). The EGFR is a member of the ErbB cell surface receptors, which are all receptor tyrosine kinases (RTKs). In 1959 researchers tried to find an oncogene that causes neuroblastoma, this gene was identified as neu (Schechter et al. 1984). The neu oncogene is homologous comparable to v-erbB, this is a viral oncogene which can be
isolated from an erythroblastosis retrovirus, this virus actually encodes a fragment of the chicken EGFR. If a bird is infected with this virus, it can cause cancer (Schechter et al. 1984). Because of this phenomenon the researchers thought that a mutated EGFR could cause cancer. Later in 1965 Cohen and colleagues discovered the epidermal growth factor (EGF) and the role of its receptor, which is to activate gene transcription and control the cell cycle progression (Cohen 1965).

The EGFR receptor consist of an intra- and extracellular domain; the active receptor can communicate through complex pathways in the cytoplasm. The EGFR and other ErbB receptors can form dimers, this is a state in which the receptor is normally active (Zhang et al. 2007). The dimerization process requires a ligand-receptor interaction, after the dimerization the intrinsic protein-tyrosine kinase domain of the receptor is stimulated. Because of this ATP can bind the lysine residue of the tyrosine kinase domain and therefore provides an organic phosphate group. This is followed by an auto phosphorylation process of several other tyrosine kinase domains on the intracellular part of the receptor. This process will induce the RAS, AKT and other transduction pathways (see also Figure 1) (Dawson et al. 2005). The above-mentioned pathways are responsible for several key regulatory cell processes, for instance angiogenesis, inhibition of apoptosis, cell proliferation and migration/invasion properties (Oda et al. 2005). Most of the time the mutations in the EGFR of NSCLC patients are heterozygous, furthermore the mutant allele can be overexpressed because of gene amplification. The EGFR L858R point mutation in exon 21 and the deletion of exon 19 are both in the intracellular catalytic domain of the EGFR receptor. These mutations are the most common and can cause activation of the receptor (Soh et al. 2009).

**How did they treat NSCLC in the past?**
The way NSCLC patients are treated is depending on the tumor stage and patient condition. Stage I, II and IIIA patients are treated with radiation, cytotoxic chemotherapy and surgery (Bulzebruck et al. 1992). Unfortunately this approach is in a lot of cases not enough to eradicate the tumor in advanced NSCLC. Small molecule EGFR-TKI’s like erlotinib (Tarceva®) and gefitinib (Iressa®) are common drugs for patients with a mutated overactive EGFR. These drugs can compete with ATP for the binding sites on the tyrosine kinase domain of the receptor (Figure 2A). As a consequence the phosphorylation and activation of the downstream signaling pathways of the EGFR are disrupted. A double-blinded phase III trial experiment between a placebo and both erlotinib and gefitinib proved that these drugs have a positive effect on the survival of Asian and Caucasian NSCLC patients, because these groups of patients usually have the proper EGFR mutation (Chang et al. 2006, Sheikh, Chambers 2013). Moreover, compared to classical cisplatin-based chemotherapy both drugs have a significant higher efficacy, an extended progression free survival (PFS) from 8.4 to 13.1 months and a better quality of life (Zhou et al. 2011). These two drugs are most commonly used in the case of the L858R point mutation in exon 21 or the deletion of exon 19. Despite the fact that these drugs seem to be quite effective, only a selected group of patients have high response rates.
Clonal selection cells with the mutation survive because of present without EGFR. Half of the tumors that were treated with (does have substitution, the EGFR methionine residue (T790M). Because of this threonine residue at location 790 with a bulky is the resistance for EGFR mutations for an NSCLC cell to develop take it over. There are several important inhibited by a drug, other pathways can simply take it over. There are several important mutations for an NSCLC cell to develop resistance for EGFR-TKI’s. The most common is the T790M mutation, this mutation replaces threonine residue at location 790 with a bulky methionine residue (T790M). Because of this substitution, the EGFR-tyrosine kinase pocket has higher affinity for ATP, resulting in a decreased affinity for erlotinib and gefitinib (Figure 2B, 2C). The T790M mutation occurs in half of the tumors that were treated with EGFR-TKI’s, the mutation is usually already present without EGFR-TKI treatment, only the cells with the mutation survive because of clonal selection. However, some studies suggest this mutation can originate because of the treatment (Kobayashi et al. 2005). To come up with a solution researchers came up with irreversible EGFR-TKI’s, these small molecules can bind covalently to a cysteine residue of the EGFR-tyrosine kinase pocket. Unfortunately most irreversible EGFR-TKI’s have many severe side effects like skin rash, stomatitis, nail loss and diarrhea. Afatinib (Giotrif®) is the most promising irreversible EGFR-TKI’s while the side effects are less compared to other experimental irreversible EGFR-TKI’s which seem to be less specific and influence other tyrosine kinase receptors as well (Tao et al. 2014).

This is for instance the case with East Asians, non-smokers and female patients. In a normal population the response rate is just about 10%. Additionally, studies have shown that there are many other important factors that can influence EGFR-TKI’s sensitivity (Fukuoka et al. 2003, Miller et al. 2004).

RESISTANCE OF EGFR-TKIs
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receptors play an important role in the activation of the RAS-Erk1/2 pathway. Hepatocyte growth factor (HGF) is the ligand of MET and it can activate the receptor through an adaptor protein (Gab1). This triggers resistance to reversible EGFR-TKI’s like erlotinib and irreversible EGFR-TKI’s like afatinib. Because the EGFR downstream signaling pathway is bypassed through the MET pathway (Turke et al. 2010, Yamada et al. 2010, Yano et al. 2008). In a Japanese study with mutant EGFR patient’s researchers observed that HGF was overexpressed in 61% of the tumors with acquired resistance. This indicates that HGF can be a useful target in combination with EGFR-TKI’s to prevent resistance (Yano et al. 2011).

Thirdly, activation AKT downstream pathway is possible because of the loss of the tumor suppressor gene PTEN. Normally this gene is responsible for the conversion of PIP₃ to PIP₂. However if PIP₃ levels stay elevated, Akt can be activated without EGFR activity. This will result in cell growth and inhibition of apoptosis (Figure 3) (Yamamoto et al. 2010).

**ALK/ROS1-RECEPTOR.**
Anaplastic Lymphoma Kinase (ALK) is a tyrosine kinase receptor of the insulin family, it is an important receptor in the central nervous system where its expression levels are low (Iwahara et al. 1997). Through the RAS, PLCγ and STAT pathways ALK is involved in cell division and cell proliferation (Figure 1). Changes in ALK can give rise to a permanent active ALK complex. In 3-4% of the cases NSCLC is caused by a chromosomal translocation of ALK and the echinoderm microtubule-associated protein-like 4 (EML4); a protein necessary for the formation of microtubules. This translocation will fuse the 3’ kinase domain of ALK with ELM4 and its promoter, as a result the translocation generates an ELM4-ALK fusion protein, which leads to ALK activation (Selinger et al. 2013). Therefore ALK will be present in relative high concentrations in all kinds of tissues, while it is generally only present in the central nervous system (Jiang et al. 2013). Furthermore, because the fusion disturbs the coiled coil domain of the receptor, ELM4-ALK is able to dimerize without the ligand of ALK. This process can lead to a signal independent kinase activity, which can inhibit apoptosis or stimulate cell proliferation and division. The above-mentioned mechanism is the most common type of ALK activation in NSCLC patients (Selinger et al. 2013). Although the cause of the underlying mechanism is completely different, both the ALK mutation and EGFR mutation have the same pathology. For this reason it is not possible to identify the cause of NSCLC just with the clinical picture. Because there are two groups of patients with different targets it is important to have a thorough test to decide whether a patient has ELM4-ALK or EGFR related NSCLC, more about this in the diagnostic section.

Another receptor that can play a role in NSCLC is the ROS1 receptor. Like the ALK receptor ROS1 is a tyrosine kinase insulin-like receptor, which can form fusion proteins as well. Approximately 1.6% of the patients have NSCLC due to a fusion in ROS1. There are 14 different genes for the fusion with the ROS1-terosine kinase domain that can lead to NSCLC. The most common is the SDC4-ROS1 fusion, SDC4 is generally a trans- membrane structure that is involved in the attachment of extracellular structures (Stumpfova, Janne 2012). Like the EML4-ALK fusion ROS1 fusions are oncogenic because the receptor can dimerize without ligand. Moreover, some fusions deliver a stronger promoter, that can lead to a higher transcription rate. This can result in high receptor concentrations in both ALK and ROS1.

**DIAGNOSTICS OF NSCLC**
For an effective NSCLC treatment it is important that the molecular background of the patient is thoroughly examined. This is because ALK related NSCLC has the same pathology compared to EGFR related NSCLC. If, for example, a patient has a mutated EGFR receptor, an ALK-TKI drug will obviously not work. An effective method to test for AKL-translocation is ‘break apart fluorescent in situ hybridization’ (FISH). Both genes are positioned on the short arm (p) of chromosome 2: EML4 is located on 2p21 and ALK is located on locus 2p23 (Figure 4).
technique uses two locus specific probes (LSP) with different colors. A red probe binds to the last (3’ end) part of the ALK gene, the green probe covers a piece of the enhancer-promoter region of the EML4 gene (Shaw et al. 2009). If a NSCLC patient has an EML4-ALK fusion gene because of break resulting in a translocation or inversion, it is easily recognized with a fluorescent microscope. This will be visible as separate red and green dots. If the probes are close together, it is often visible as a yellow dot (Figure 4E). If more than 50% of the cells have a chromosomal translocation pattern, the patient is EML4-ALK positive. If, however, less that 10% of the cells are negative, the patient is considered EML4-ALK negative.

When the results of the positive cells are >10% and <50%, another 50 cells are scored. When 15% of these cells shows a chromosomal translocation, the test is considered positive (Teixido 2014). Similarly, ROS1 translocations can be identified with break apart FISH as well. Because of this arbitrary cutoff of 15% some patients are classified as EML4-ALK or SDC4-ROS1 negative. In the past a patient with 13% positive cells was regarded as EML4-ALK negative while being EML4-ALK positive. In addition, if an inversion involves a small locus it could result in a false negative as well (Soda et al. 2007).

A second technique is the use of IHC, there are several antibody’s (ALK1, 5A4 and D5F3) on the market with the same specificity compared to a break apart FISH test (Figure 4B, 4C) (Selinger et al. 2013). Although it is a promising and easy technique, the use of IHC is unfortunately not commercially available in a lot of countries like the US because it is not approved by the food and drug administration (FDA). It is also possible to screen NSCLC patients with RT-PCR, this is a rapid and cheap identification method for each unique known variant. The problem with this technique however is that a specific probe is required. For this reason unknown variants will not be detected (Fu et al. 2014). The the most effective method for the screening of a translocation seems to be IHC, especially when it is commercially available in all countries.

**EGFR-TKI RESISTANCE: NEW APPROACH**

To overcome the resistance against EGFR-TKI’s and researchers came up with new generation of promising EGFR-TKI’s like crizotinib (Xalori®), afatinib and WZ4002. Crizotinib is capable of inhibiting the ALK, ROS1 and MET receptors. Like other TKI’s crizotinib competes with ATP for the binding sites on the tyrosine kinase domain of these receptors. This drug is already been used for the treatment of EML4-ALK NSCLC patients and is approved by the FDA since August 2011 (Ou et al. 2012, Rodig, Shapiro 2010). WZ4002 is a third generation mutant-selective irreversible EGFR-TKI.
PROTO-ONCOGENE TYROSINE-KINASE: SRC
Recent studies showed that the previous mentioned mutations like the gatekeeper and MET amplification are not the only ways a NSCLC tumor can acquire resistance to gefitinib and afatinib. There are several other alternative kinases that can activate the downstream signaling pathways. The Proto-oncogene SRC for instance is a non-receptor tyrosine kinase that is capable of phosphorylating many other proteins (Wheeler, Iida & Dunn 2009). One of the proteins that can be activated by Src is the intracellular part of the MET-receptor. Therefore HGF-independent activation of the MET receptor is possible through Src activation, this is commonly seen in erlotinib resistant cells (Stabile et al. 2013). In addition, Src is capable of activating the AKT, RAS and STAT pathways. To block these downstream signaling pathways a Src inhibitor can be used. Moreover, Src can inhibit Anoikis. This is a form of programmed cell death like apoptosis, it’s normally mediated through the detachment of the cell and the extracellular matrix in anchorage-dependent cells. This process can inhibited by Src. Because of Src activation the lung tumor can become resistant to anoiksis (Sakuma et al. 2010). A Japanese study showed that WZ4002 is able to block the Scr pathway more efficiently compared to Src inhibitors in lung adenocarcinomas and other types of NSCLC. This was tested with the HCC827 (del 747-750) and the H1975 (double mutation: T790M/L858R) cell lines (Sakuma et al. 2012).

EFFICACY OF THE NEW GENERATION EGFR-TKI AND CRIZOTINIB DUAL TREATMENT
To compare the effect of crizotinib alone or in combination with afatinib and WZ4002, Japanese researchers used different NSCLC cell types (Nanjo et al. 2013). The first cell line is an EGFR mutant with a deletion in exon 19 resulting in a high expression of the EGFR receptor. Secondly, MET amplification cells were used, these cells have the same deletions with a MET gene amplification (Suda et al. 2010). Thirdly, the researchers used NSCLC cells with the T790M mutation. The cells were exposed to increasing concentrations of EGFR-TKI’s: afatinib and WZ4002. At the same time the cells were exposed to crizotinib (ALK, ROS1 and MET inhibitor), HGF and the combination of crizotinib and HGF while the cell proliferation was monitored. In both the cell lines crizotinib and afatinib reduced proliferation. However, the external added HGF generated EGFR-TKI resistance, this process could be reduced by the use of an EGFR-TKI and crizotinib. Another experiment showed that the gefitinib resistant cells were sensitive to afatinib and WZ4002. Although HGF was exogenously added, crizotinib still sensitized those cells. Furthermore, the combination of crizotinib and a new generation EGFR-TKI reduced cell proliferation significantly in the MET amplified cells even with exogenously added HGF.

These experiments indicate that the combination of crizotinib plus WZ4002 or afatinib can prevent EGFR-TKI resistance in NSCLC cells with MET receptor amplification, HGF overexpression and the T790M mutation (Nanjo et al. 2013). Secondly WZ4002 is about 30 - 100 times more potent compared to the currently used EGFR-TKI’s like afatinib and erlotinib, especially against the T790M EGFR mutant cell lines (Sakuma et al. 2012).

To examine the efficacy of crizotinib and the new generation EGFR-TKI’s (afatinib, WZ4002) in vivo, mouse models were used in another experiment. NSCLC cells with the T790M mutation and the same cells with a HGF gene transfection were injected in immunodeficiency (SCID) mice. As a result the mice developed tumors. The most successful treatment of the T790M HGF transfected cells was observed with the combination of crizotinib and WZ4002 or afatinib. A treatment with just crizotinib, afatinib or WZ4002 alone was not effective with these cells. The administration of just afatinib or WZ4002 inhibited growth significantly in the cells with just the T790M mutation, however the administration of only crizotinib was not effective. This strongly suggests that the resistance against EGFR-TKI is induced by the expression of HGF in vivo (Nanjo et al. 2013).
TOXICITY
The above results show that both afatinib and WZ4002 are quite effective in combination with crizotinib. However, a lot of EGFR-TKI's can induce severe side effects. This is especially the case in high concentrations or in combination with other drugs. Afatinib for instance can causes diarrhea and acneiform (acne like skin condition) in more than 30% of the patients. Some side effects of EGFR-TKI's like deceased appetite, itching, weight loss and nose bleeds are less common, they occur in about 10-29% (Tao et al. 2014). To test the toxicity of a dual treatment of afatinib or WZ4002 with crizotinib during the in vivo experiment, Najo and his group measured the mice body weight. The intestinal mucosal damage was analyzed as well.

The treatment of the three agents alone and the combination of WZ4002 and crizotinib had a minor effect of intestinal damage or bodyweight. However, the combination of afatinib and crizotinib cause a massive drop in bodyweight. Furthermore, this combination induced severe intestinal mucosal damage (Nanjo et al. 2013).

DISCUSSION
The use of crizotinib is already successful with EML4-ALK NSCLC patients. In addition, mouse models and cell culture experiments show that the combination of a new generation EGFR-TKI's (afatinib or WZ4002) with crizotinib can overcome resistance to reversible EGFR-TKI's like erlotinib and gefitinib. These results suggest that the blockage of both the mutant EGFR and MET ecteors by the dual treatment of crizotinib, and new the generation EGFR-TKI's can be quite promising. Because the combination of afatinib and crizotinib can induce severe side effects the combination of crizotinib and WZ4002, which is less toxic, is more favorable. Furthermore the Src pathway that is blocked by WZ4002 can actually play an important role as well. In the future proper clinical experiments are needed to test this combination in humans. The personal approach to treat different types of NSCLC can only be performed if the molecular characteristics are fully known. This can be accomplished through the use of IHC or a ‘brake apart FISH’ analysis.
REFERENCES


