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Relevance of detection the mechanisms of resistance to ALK TKIs in ALK positive NSCLC

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Abstract
NSCLC is the most common type of lung cancer and can be associated with rearrangement of the anaplastic lymphoma kinase (ALK). Translocation of ALK with fusion partners leads to continuous activation of downstream signaling pathways involved in proliferation and anti-apoptosis. ALK positive NSCLC patients are treated with tyrosine kinase inhibitors (TKIs), however most patients become resistant and develop a relapse. On-target and off-target mechanisms contribute to resistance in ALK positive NSCLC. For on-target mechanisms, L1196M is thought to be the main driver of crizotinib resistance, while G1202R is thought to be the main driver of alectinib resistance. It remains unclear whether ALK resistance mutations, specifically L1196M, can be detected in treatment naive tumors. However, monitoring concentration of both L1196M and G1202R, by taking liquid biopsies predicts a patients' clinical course and may improve treatment options. ddPCR seems to be the most effective tool to detect ALK TKI resistance mutations in liquid biopsies, but when a larger gene panel must be investigated, NGS seems to be a better tool. When no on-target resistance mechanisms can be found, it should be considered to analyse any off-target resistance mechanisms. Activation of the by-pass signaling pathway EGFR in ALK positive patients, contributes to resistance and may even be detected before start of treatment. Better treatment can be given when the mechanism of resistance in an ALK positive NSCLC patient is well understood, which therefore improves a patients' clinical outcome.

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Introduction

Lung cancer is the second most common cancer in both women and men and is the leading cause of death worldwide, with about 2.2 million new cancer cases and 1.8 million deaths in 2020 (Sung et al., 2021). Lung cancer can be divided into two categories which are treated very differently. Small cell lung cancer SCLC is one of the types and accounts for about 10-15% of all lung cancers and is sometimes called oat cell cancer. The most common type is non-small cell lung cancer (NSCLC), which accounts for about 80% of all lung cancers. Within this type of lung cancer there are subtypes, based on in which type of lung cell the cancer starts, this includes adenocarcinoma, squamous cell carcinoma, large cell carcinoma and two other types which are less common called adenosquamous and sarcomatoid carcinoma (American Cancer Society, 2021). 65% of patients with NSCLC show locally advanced or metastatic disease (Reck et al., 2013). NSCLC is associated with smoking, therefore patients that are diagnosed are almost always smokers or former smokers. However, there is also a percentage of patients diagnosed with NSCLC that never smoked, which suggests that genetics and environmental factors play a role in these patients (Johnson et al., 2012).

Looking at the molecular level, mutations in epidermal growth factor receptor (EGFR) tyrosine kinase are often found in patients that have never smoked. Also, other mutations present in KRAS and TP53 are commonly found in NSCLC patients. Improvement in sequence techniques in the past years, increased insight into the genomes of tumors (Liao et al., 2015). This also led to the identification of gene rearrangement of the anaplastic lymphoma kinase (ALK) (Iacono et al., 2015), which was first discovered by Soda et al. in 2007 (Soda et al., 2007). These rearrangements include inversion or translocations of ALK with fusion partners, which leads to continuous activation of downstream signaling pathways involved in proliferation and anti-apoptosis (Iacono et al. 2015) (Le et al., 2017). Therefore, ALK rearrangements are identified as oncogenes and play a key role in the pathogenesis of NSCLC (Soda et al., 2007). Many ALK fusion variants are described, but the most common one is the microtubule-associated protein-like 4-anaplastic lymphoma kinase (EML4-ALK). ALK encodes a receptor protein tyrosine kinase and EML4 encodes a microtubule-associated protein, which contains a coiled-coil domain. Both are located on chromosome 2, on the same short arm (Mano, 2015). Due to an inversion rearrangement from inv.(2) (p21;p23), EML4 is directly fused to the intracellular kinase domain of ALK (Mano, 2015)(Du et al., 2018). ALK undergoes dimerization mediated by the coil-coiled domain of EML4, which leads to constant activation of the tyrosine kinase function of ALK, therefore ALK rearrangements are identified as oncogenes. Several variants of the EML4-ALK exist, because EML4 contains different breakpoints. These variants and their frequencies are V1 (54.5%), V2 (10%), V3a/V3b (34%) and V5a (1.5%) (Du et al., 2018). In most cases the breakpoint within the ALK gene lies near the 5' end of exon 20 (Bayliss et al., 2016). Generally, it is estimated that 3%-7% of the NSCLC patients have an ALK rearrangement (Dagogo-Jack et al., 2016), but it appears more often in younger patients that show adenocarcinoma histology and never smoked or light smokers (Du et al., 2018).

Knowing whether a NSCLC patient is ALK positive is critical for treatment, therefore it is nowadays recommended to test all NSCLC patients with an adenocarcinoma component for presence of ALK rearrangements by taking a biopsy. Methods used to detect ALK rearrangement in these biopsies are usually fluorescence in situ hybridization (FISH), immunohistochemical (IHC), reverse transcriptase-PCR (RT-PCR) and next generation sequencing (NGS). FISH is used to be the gold standard to detect whether a patient is ALK positive (Du et al., 2016), however IHC assays are nowadays

more often used (Cabillic et al., 2018). Treatment of NSCLC has changed in the past decades and a great success was achieved in the early 2000's with conventional chemotherapeutic drugs. At this time, the median survival was about 12 months and somewhat longer in clinical studies. This progression was supported by the introduction of new drugs and by improved recognition of the biology of these cancers based on the molecular profile. These so-called driver genes allowed a reliable prediction of drug effectiveness (Reck et al., 2013). ALK rearrangements in NSCLC are generally treatable with tyrosine kinase inhibitors (TKI). Crizotinib was the first ALK TKI to enter clinical development, which showed remarkable responses in ALK positive NSCLC patients (Kwak et al., 2010). It improved survival of patients and became standard treatment for patients with advanced ALK positive NSCLC (Gainor et al., 2016), however most patients become resistant and develop a relapse (Takeda et al., 2013). Luckily, since the approval of crizotinib, other ALK TKIs have been developed. These inhibitors overcome the resistance mechanisms which were induced due to crizotinib treatment. Many mutations that lead to acquired resistance have been described, but whether these mutations were already present before start of treatment remains unclear. NSCLC patients with mutations in the epidermal growth factor receptor (EGFR) are treated with EGFR TKIs. These patients also relapse after an initial good response. Resistance is frequently associated with gain of an EGFR T970M gatekeeper mutation. Presence of this mutation at low frequency was shown in biopsies obtained before start of treatment with a TKI (Toyooka et al., 2005) (Chiang et al., 2020). Understanding EGFR mutations may predict clinical outcome and allows patients to obtain personalized targeted therapies and prevent relapse (Nan et al., 2017) Whether this is also the case for ALK TKI resistance mutations is unknown, therefore I want to answer the following questions: Can mutations that lead to ALK TKI resistance in ALK positive NSCLC patients be detected before start of treatment and would it be relevant to test this in routine clinical practice? To answer these questions, in this paper I will describe the mechanisms of ALK TKI resistance and what they found in *in vitro* and *in vivo* studies. Also, I will discuss the techniques used to discover and monitor the genomic composition of a tumor and what has been done in previous studies and the relevance of it.

Mechanisms of resistance

On-target mechanisms

Several resistance mechanisms have been identified ever since ALK has been a target in NSCLC. These resistance mechanisms can be classified into two categories, including on-target or 'ALK-dependent' and off-target or 'ALK-independent' (Niu et al., 2017). On-target mechanisms are usually genetic modifications in the kinase domain and off-target mechanisms are dysregulation of by-pass signaling pathways or mechanisms of drugs-efflux (Tabbò et al., 2020). Identification of the mechanism that leads to ALK TKI resistance is important for proper treatment targeted at the underlying resistance mechanism (Niu et al., 2017). For on-target mechanisms of resistance, many mutations within the ALK domain are identified. However, the mutations differ greatly depending on the type of ALK TKI that was given and on tumor biology. Once a tumor cell with a mutation in the kinase domain has a fitness benefit, this cell will survive and grow and become a dominant clone at relapse (Friedman, 2016). As said, different TKIs can lead to different genetic on-target alterations. ALK TKIs can be divided into first, second and third generation inhibitors with each have their own modes of action.

Off-target mechanisms
Besides on-target mechanisms of resistance, off-target target mechanisms can also lead to TKI resistance in ALK positive patients. These mechanisms include activation of bypass tracks and lineage changes, so tumor cells become independent of ALK. Resistance caused by on-target mechanisms, which I have described above, only account for 30% of crizotinib resistance in ALK positive NSCLC patients. Most resistant tumors harbor an ALK-independent, also known as off-target, resistance mechanisms, by the activation of bypass signaling tracks, autocrine signaling, genetic alterations or dysregulation of feedback signaling. This all results in reactivation of downstream effectors necessary for tumor growth and survival (Lin et al., 2017).

Detection of on-target mechanisms of resistance

Crizotinib resistance in vitro
Crizotinib is a first-generation inhibitor and patients usually relapse within one to two years when treated with crizotinib (Tabbò et al., 2020). The mechanism of crizotinib resistance was investigated by katayama et al., in a NSCLC cell line (Katayama et al., 2011). In the H3122 cell line, EML4-ALK variant 1 was expressed which is sensitive for treatment with crizotinib. Resistant clones were generated by exposing sensitive parental cells to increasing concentration of crizotinib. It was observed in the Western blot analysis that the H3122 crizotinib-resistant (H3122-CR) showed AKT and ERK phosphorylation and higher protein levels of EML4-ALK, compared to the parental H3122 cells. Both observations are associated with cell growth and survival (Katayama et al., 2011).

FISH was performed to detect DNA copies to investigate if amplification of ALK might be responsible for the increased protein levels observed in the Western blot. This analysis showed an increased number of rearranged EML4-ALK genes per cell in H3122 CR cells compared to parental cells. In a different study of Katayama et al., they found that amplification of wild-type EML4-ALK in H3122 cells causes resistance to crizotinib (Katayama, 2018). However, phosphorylation of EML4-ALK in H3122 CR cells was not affected by treatment with 1μM crizotinib dose, which suggests these cells harbor a mutation within ALK, besides amplification of EML4-ALK. Next, the coding sequence of the EML4-ALK was analyzed at the transcript level in H3122-CR and the parental cell line. A C-A substitution in nucleotide 3586 was observed in H3122 CR cells and not in parental cells. This substitution leads to a L1196M substitution, also known as an ALK gatekeeper mutation (Katayama et al., 2011).

Engineered Ba/F3 cells that harbor this mutation, conferred resistance to crizotinib, but wild-type EML4-ALK did not. Western blot analysis showed increased ALK protein level and p-ALK in parental cell lines and in the cells with a mutant EML4-ALK fusion gene, but not in the cells with the unmutated EML4-ALK. To detect whether L1196M was present in these cells, a highly sensitive gate-keeper mutation specific PCR assay was used. This assay can detect mutations when it represents only 1% of the mutated ALK alleles. This was also used in the H3122CR and parental cells, to compare the different cell lines. The PCR assay showed presence of L1196M in H3122 CR cells, but not in the other two cell lines. This suggests that there might be an order in developing acquired resistance, involving amplification of a target first when exposed to a lower dose of crizotinib (600 nM), followed by a secondary gatekeeper mutation which makes cells resistant to 1 μM crizotinib (Katayama et al., 2018).

Crizotinib resistance in vivo
In a study of Gainor et al. they investigated ALK resistance mutations *in vivo* (Gainor et al., 2016). 51 ALK positive patients progressing on crizotinib were included, from which repeated biopsies were taken from progression lesions. All biopsies were analysed for ALK resistant mutations using targeted NGS and Sanger dideoxynucleotide sequencing of complementary DNA (cDNA) and genomic DNA (gDNA). NGS was used to detect single-nucleotide variants (SNVs) and deletion/insertions within the gene of interest. Sanger sequencing was used in some specimens to sequence the entire ALK kinase domain. In 11 specimens they found ALK resistance mutations, the most common were L1196M and G1269A. L1196M is known as a gatekeeper mutation, since it sterically blocks the binding of the drug (Kay et al., 2017). G1269A is a mutation that alters the ATP binding pocket, therefore alters the conformation, and prevents binding of a TKI (Tabbò et al., 2020). From these specimens, 36 were tested for ALK rearrangement and amplification by FISH. All showed ALK rearrangement, and 3 specimens showed ALK gene amplification. Specimens with ALK gene amplification did not harbor ALK resistance mutations, so 31% of all specimens showed on-target genetic alterations, either ALK amplification or ALK resistance mutations (Gainor et al., 2016).

Resistance to second-generation TKIs
In the same study, 23 patients treated with certinib, biopsies were taken and were analysed for ALK resistance mutations. 24 biopsies were taken in total, in which secondary G1202R and F1174C/L mutations were detected in more than half of the specimens. Also, G1202del was found in 8% of the specimens. In engineered Ba/F3 cells expressing EML4-ALK with a G1202del they showed that this mutation confers resistance to certinib, alectinib and brigatinib, but the effectiveness of crizotinib was less impacted by this mutation. The G1202del has in contrast to G1202R, which confers resistance to all first and second-generation ALK inhibitors, a different effect (Gainor et al., 2016.

The next cohort evaluated 17 patients that progressed on alectinib, after first receiving crizotinib. In over a half of the alectinib-resistant biopsies ALK resistance mutations were found, where G1202R was the most common. Also, the V1180L mutation was found, which was the first clinical observation of this mutation in an alectinib-resistant patient. Although alectinib seemed to be effective against the L1196M mutation in previous studies (Sakamoto et al., 2011) (Song et al., 2015), in this study this was only the case for one patient. The next second-generation ALK inhibitor analysed is brigatinib. Most of the patients treated with this inhibitor were previously treated with crizotinib. In 5 out of the 7 patients ALK-resistant mutations were found, with the G1202R being the most common one (Gainor et al., 2016).

Resistance to third-generation TKIs
Luckily, resistance mutations that occurred on progression with a secondary ALK inhibitor can be overcome by a third-generation inhibitor called lorlatinib. Ba/F3 cells were engineered to express EML4-ALK (E13;A20) wild-type or EML4-ALK that harbors various ALK mutations that confer resistance to second-generation ALK inhibitors. These cells were treated with crizotinib, ceritinib, alectinib, brigatinib and lorlatinib. All mutant variants showed a good response to lorlatinib, including the G1202R. In a study of Yona et al. biopsies of 20 patients with ALK positive lung cancer relapsing on lorlatinib were studied. Most of the patients previously received crizotinib and a second-generation ALK-inhibitor. Barely any patients that conferred primary intrinsic lorlatinib resistance carried ALK mutations. This may be explained by ALK-independent mechanisms that lead to resistance. Contrarily, patients with acquired mutations appeared to frequently have compound mutations (Yoda et al., 2018). Compound

mutations observed were ALK-G1202R/L1196M, ALK-E1210K/D1203N/G1269A, ALK-I1131N/L1198F. The G1202R/L1196M compound mutation was the most common mutation found in patients treated with lorlatinib. Unfortunately, patients with a G1202R compound mutation are resistant to all available ALK-TKI, which severely limits the treatment options for these patients (Sharma et al., 2019).

Relevance of ALK resistance mutations detection

Detection of pre-existing crizotinib resistance mutations
As stated before, the gatekeeper mutation L1196 is a driver of resistance to crizotinib (Tabbò et al., 2020)) (Gainor et al., 2016). In a study of katayama et al., biopsies were taken from 18 patients after they relapsed on crizotinib (Katayama et al., 2012). Duration of crizotinib treatment varied between 4 to 34 months. Biopsies were taken from mediastinal lymph nodes or malignant pleural effusions. From most of the patients repeated biopsies were taken. FISH was used to confirm the ALK fusion gene, which was positive in almost all patients. The presence of mutations after treatment was investigated, and they also investigated whether ALK mutations were present before start of treatment treatment. In 3 patients with resistant mutations, pre-crizotinib treatment specimens were obtained. They used Sanger sequencing to discover any mutations in the pre-crizotinib samples, but no mutations were detected. They further investigated the pre-crizotinib resistant samples by using allele-specific PCR assay, however this was only done for one patient (Katayama et al., 2012). This type of essay has previously been done in a different study of Katayama et al. and it is able to detect L1196M mutation when it constitutes no less than 1% of the mutated ALK alleles. It is a highly sensitive assay with a detection limit of 0.3% or less (Katayama et al., 2011). This assay identified L1196M in the resistant specimen, but not in the pre-crizotinib specimen (Katayama et al., 2012).

Monitor ALK resistance mutations
In a study of Yoshida et al. they studied presence of ALK-resistance mutations in 7 ALK positive patients that undergo treatment or have been previously treated were investigated (Yoshida et al., 2018). Patients were diagnosed with ALK positive NSCLC by either FISH or IHC. Blood samples were taken every 3 months during treatment with an ALK inhibitor. Since the use of NSG is time consuming and expensive, they used ddPCR to detect secondary mutations in ALK positive patients. This technique has been applied to detect KRAS mutations and DNA copy number alterations, and it has been shown that ddPCR has a high sensitivity for detecting mutations even at frequencies as low as 0.05-0.01% (Pinheiro et al., 2012) (Taly et al., 2013). ddPCR assays are based on negative or positive droplets, from which absolute DNA quantity can be calculated (Rowlands et al., 2019). Using ddPCR, ALK resistant mutations were detected in circulating free DNA (cfDNA) present in plasma. They analyzed 10 G1296A, L1152R, F1174L, V1180L, L1171T, G1202R and S1206Y. This study also described the

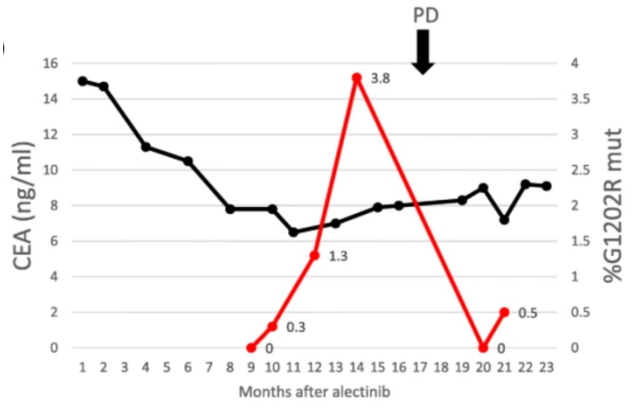

Figure 1. Clinical course of patient on alectinib by monitoring G1202R levels with ddPCR. Yoshida, R. et al. (2018). Highly sensitive detection of ALK resistance mutations in plasma using droplet digital PCR. BMC Cancer 18, 1136. http://creativecommons.org/publicdomain/zero/1.0/. No changes were made.

clinical course G1202R positive NSCLC, so they confirmed the sensitivity and specificity of this mutation using ddPCR. Preliminary experiments were performed using ALK wild-type and mutant samples and a limitation of 0.01% was found for G1202R. All patients, except one, were already treated with either crizotinib or alectinib. cfDNA analysis showed that 2/7 patients were positive for ddPCR. In both cases the G1202R mutation was observed, however one was treated with alectinib and the other with crizotinib. Some other tumors will still be controlled, but these were also scored negative for all 10 secondary mutations. From one of the two patients that were treated with crizotinib a pre-treated sample was obtained from one patient, which also scored negative for secondary mutations. For the patient on alectinib where G1202R was found in the blood sample, they also described the clinical course of this patient (Figure 1). Blood samples were taken from this patient and analysed with ddPCR, this showed the presence of G1202R after 11 months of therapy. However, the mutation-specific assay showed the presence of G1202R already after 9 months of therapy. Overall, plasma levels of this ALK mutation corresponded with the clinical course of the patient since the mutant increased before progression occurred (Yoshida et al., 2018).

In a study of Madsen et al. they investigated the genomic composition of ALK positive NSCLC prior to and following ALK-TKI treatment to predict treatment outcome 24 patients with advanced ALK positive NSCLC were included in the study. 14 of the 24 patients were treatment naive. Genomic profiles were analysed by taking cfDNA samples before treatment and during treatment with ALK TKI. Using NGS with targeted deep sequencing (CAPP-Seq), alterations were detected in pre-treatment samples from 24 patients. In 55% of the pre-treatment samples genetic alterations were found. These genomic alterations include single-nucleotide variants in ALK, TP53, KRAS or other genes. Also, copy number variations in EGFR, MET and ERBB2 included. Patients with genomic alterations before treatment had significantly shorter progression-free survival (PFS), than patients where no genomic alterations were detectable irrespective of the type of ALK TKI given. Besides looking at genomic alterations prior to treatment with an ALK TKI, they also examined alterations early after start of treatment, which may predict outcome. Patients that showed alterations in samples obtained within 2 months after start of treatment, had a significantly shorter PFS, than patients that did not show genetic alterations in their cfDNA. In this study they also performed longitudinal monitoring of ctDNA SNVs, by using ddPCR. SNVs were first identified using NGS, since ddPCR has not the potential to quantify ALK rearrangements, only patients with detectable SNVs in their ctDNA were further monitored with ddPCR. During longitudinal monitoring of ctDNA, a correlation between tumor response and decreasing mutant allele frequency was found. Also, when an increase in the mutant allele frequency was observed, it was associated with progressive disease (PD). PD could be detected in ctDNA with a median timespan of 69 days, which means PD could be detected with ctDNA analysis 69 days before a patient showed PD. In this patient two ALK mutations were identified,

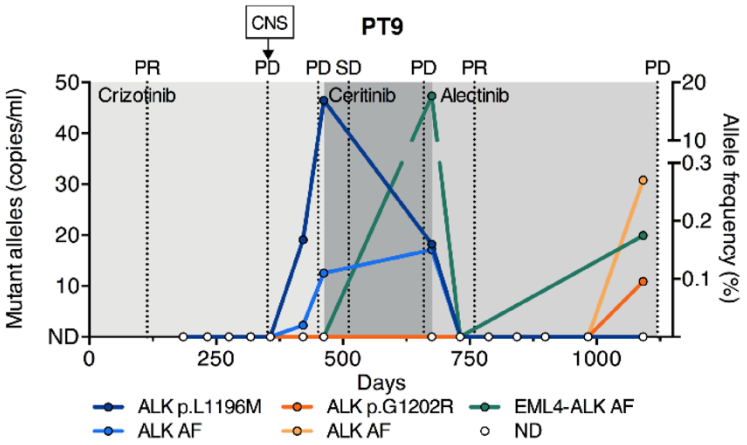

Figure 2. Clinical course of a patient by determine mutant allele levels of L1196M and G1202R using ddPCR. Madsen, A. T. et al. (2020). Genomic Profiling of Circulating Tumor DNA Predicts Outcome and Demonstrates Tumor Evolution in ALK-Positive Non-Small Cell Lung Cancer Patients. Cancers, 12(4), 947. http://creativecommons.org/licenses/by/4.0/

L1196M and G1202R. Mutant allele frequency of ALK p.L1196M starts to increase during treatment of crizotinib and before PD. The frequency of ALK p.G1202R started to increase during treatment with second-generation TKI alectinib just before PD (figure 2) (Madsen et al., 2020).

Off-target mechanisms of resistance and detection

Activation of multiple bypass signaling tracks have been described in ALK TKI resistance, with EGFR activation being the first (Lin et al., 2017). In a study of Katayama et al. they studied the role of EGFR activation in crizotinib-resistant H3122 cells, which contained the EML4_ALK fusion gene (Katayama et al., 2012). An engineered cell line which expresses EML4-ALK variant 1 became resistant to crizotinib, since they were exposed to increasing concentration of crizotinib for more than 4 months. No ALK resistance mutation or EM4-ALK gene amplification was present in this cell line. Crizotinib suppressed phosphorylation of ALK, but AKT and ERK activation was still maintained, which suggests these pathways are maintained by other regulators than ALK. Using phospho-RTK assays, increased EGFR phosphorylation was found in crizotinib-resistant cell lines, compared to crizotinib-sensitive cells. Also, higher EGFR mRNA expression was found and activation of downstream ERK and AKT signaling. This suggests that EGFR activation plays a role in crizotinib resistance. To confirm this, the authors also studied EGFR amplification in patient specimens. In 18 patients that relapsed on crizotinib an IHC staining was done to detect phosphoEGFR. In 4 of the 9 post-treatment specimens, increased EGFR activation was found in the resistant sample compared to the corresponding pre-treated sample. This suggests that EGFR might play a role in crizotinib resistance in ALK positive patients. Looking at the pre-treated samples, all cases, except for one, showed EGFR activation. To determine whether EGFR activation is important in crizotinib resistance, they looked at a primary culture of a patient. The cells were crizotinib-naive and ALK positive and showed high levels of phosphorylated EGFR. The combination of crizotinib and gefitinib treatment, which is an EGFR inhibitor, led to marked inhibition of downstream ERK and AKT phosphorylation. So, the combination of an ALK inhibitor and EGFR inhibitor leads to more growth suppression and increased activation of apoptosis. This suggests that EGFR activity contributes to the maintenance of downstream signaling and thereby crizotinib resistance, which can already be detected as an intrinsic resistance mechanism before start of treatment with crizotinib (Katayama et al., 2012).

Conclusion and discussion

L1196M mutations are thought to be the main driver of crizotinib-resistance, looking at the *in vitro* and *in vivo* studies (Katayaman et al., 2011)(Gainor et al., 2016). Besides, it is analogous to T970M in EGFR, which is also a gatekeeper mutation and the driver of acquired resistance in EGFR positive lung adenocarcinomas (Choi et al., 2010).
For the study of Gainor et al., a relatively large cohort was used, where in total 103 biopsies were observed. This study gives therefore a good clinical representation about the type of mutations that confer resistance to specific TKIs. Overall, it was found that secondary mutations are more common after treatment with a secondary inhibitor compared to crizotinib and the most common resistance mutation found after receiving secondary inhibitors is G1202R. Despite L1196M was observed after treatment with crizotinib, it could not be observed in pre-crizotinib samples using Sanger sequencing and an allele-specific PCR assay, in the study of Katayama et al (Katayama et al., 2012). The same was observed in the study of Yoshida et al., where one pre-crizotinib specimen was screened for T1151ins, C1156Y, L1196M, G1296A, L1152R, F1174L,

V1180L, L1171T, G1202R and S1206Y, using ddPCR (Yoshida et al., 2018). These results show that ALK mutations that confer resistance to crizotinib are not prevalent in tumors before treatment of crizotinib. However, these two studies included a total of 4 pre-crizotinib specimens, which are very few samples to draw a conclusion from. Not many studies have investigated pre-existing mutations in treatment naive specimens, so a larger cohort is needed to confirm whether pre-existing mutations are present, or not.

However, Madsen et al. studied the presence of ctDNA alterations in pre-treated samples using NGS. They investigated not the presence of specific ctDNA alterations, but only the presence of SNV or CNV. Although they did not investigate the specific ALK kinase domain mutations, it did show the impact of genetic alterations prior to treatment, since patients with alterations in their sample prior to treatment showed a significantly shorter PFS. Some patients however were already treated with some ALK TKIs, but despite which ALK TKI administered, all patients with genomic alterations had a shorter PFS (Madsen et al., 2020. These results suggest that investigation of several genetic alterations in ctDNA prior to treatment would be relevant to predict the clinical outcome of a patient and improve therapy strategy. The same accounts for monitoring ctDNA alterations during treatment, which also showed that individuals with ctDNA alterations had a shorter PFS. This importance of detecting genetic alterations will be even more confirmed in the same study, where specific ALK kinase domain mutations were monitored. ddPCR analysis showed that L1196M predicted treatment outcome of the patients treated with crizotinib, since mutant levels increased before PD (Madsen et al., 2020)

The same could be stated about G1202R levels, which has been shown to be the most common mutation to confer resistance to second-generation inhibitors, including alectinib (Gainor et al., 2016). Monitoring G1202R levels using ddPCR also showed to predict treatment outcome. The same was shown in the study of Yoshida et al., where G1202R levels corresponded to the clinical course of the patient. I did not include any studies that monitor genomic composition of a tumor treated with other second-generation, like ceritinib or brigatinib. Since the G1202R mutation is common after second-generation inhibitors in general, detection of these mutant allele levels could also predict the clinical course on TKIs like ceritinib or brigatinib. Overall, the results suggest the importance of monitoring ALK resistant mutations during the treatment of a TKI to predict a patients' clinical course and to improve treatment options (Madsen et al., 2020.

Tissue biopsies has been the standard procedure for genetic analysis of NSCLC, however analysing liquid biopsies becomes a more appealing approach. In the study of both studies (Yoshida et al., 2018; Madsen et al., 2020) they demonstrated the effectiveness of detection of ALK-TKI resistance mutations in liquid biopsies, using ddPCR. Tissue biopsy, compared to liquid biopsy, is highly invasive and is limited by tumor accessibility. Therefore, liquid biopsies may be a useful tool in the clinical practice to allow evaluation of NSCLC patients. The disadvantage of liquid biopsies is that they may contain low levels of ctDNA to detect relevant targets in NSCLC, but more research must be done into the role of liquid biopsies in NSCLC patients (Rijavec et al., 2019).

Concerning the different techniques used to detect genomic alteration in biopsies, NGS and ddPCR are the two most often used technique do detect ALK kinase domain mutations. ddPCR has a very high sensitivity and can detect mutations in biopsies at low DNA concentrations. However, a disadvantage of ddPCR is that for each mutation

that must be analysed a single assay is required. This is cost-effective if you want to analyse individual mutations, but if a larger mutational panel must be analysed, NGS is more effective (Zhang et al., 2019). In the study of Madsen et al. they first investigated SNV's using NGS, and they further monitored the detected SNV's using ddPCR. This is an effective way to monitor a larger gene panel using NGS first, since ddPCR has a limited capability to quantify structural variants. Nevertheless, ddPCR would be a useful tool to detect specific ALK kinase domain mutations that confer resistance, since the common mutations are known, so the gene panel is small (Gainor et al., 2016).

Secondary ALK mutations are an important mechanism of resistance, but 40-50% of individuals resistant to second-generation ALK-TKI harbor a different mechanism of resistance (Lin et al., 2017). The most important category of off-target mechanism is activation of bypass signaling tracks. The first described is activation of EGFR in ALK positive NSCLC. It was confirmed in an engineered cell line resistant to crizotinib, EGFR plays a role in crizotinib resistance. Also, increased phosphorylation levels of EGFR in the crizotinib-naïve specimens suggest that resistance mediated by EGFR, may be detected before start of treatment (Katayama et al., 2012). For patients that show resistance to crizotinib, but no on-target resistance mechanism can be found, it should be considered to analyse any off-target mechanisms of resistance. It would be helpful for the patient to understand the underlying off-target mechanism, so in this case EGFR inhibitors can be given, in addition to a TKI, to improve a patients' clinical course.

Resistance mechanism	*TKI to which it confers resistance*	*Found in treatment-naïve tumors*	*Type of biopsy taken*	*Techniques used for analysis*	*Able to predict patients' clinical course*
L1196M	Crizotinib	no	Liquid	ddPCR	Yes
G1202R	Alectinib	Not studied	Liquid	ddPCR	Yes
EGFR activation	Crizotinib	Not studied	Tissue	IHC staining	Not studied

Table 1. Overview of the studies of Yoshida et al. (2018), Madsen et al., (2020) and Katayama et al. (2012).

In table 1 I made an overview of the studies I included in this paper. In conclusion, it remains unclear whether mutations that confer resistance to ALK TKIs can be detected in treatment-naïve tumors. However, it would be relevant to perform longitudinal monitoring ctDNA of ALK positive NSCLC tumors to predict a patient's clinical outcome and improve treatment options. To monitor ctDNA in biopsies, especially to detect ALK kinase domain mutations, ddPCR might be a promising tool. So, understanding the mechanisms of resistance in an ALK-positive NSCLC patient, whether it is on-target or off-target, will allow better treatment options and improve a patients' clinical outcome.

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Foreword and Afterword

Since lung cancer is the second most common cancer worldwide, improve treatment options and a patients' clinical outcome is of major importance. ALK TKI resistance is a major problem, and more research must be done into how to prevent and overcome this. In this paper I wanted to highlight the importance to understand the mechanism behind resistance. When the target is known better treatment options can be given, which improve the clinical outcome. When the best treatment option is known for a patient, it will reduce the burden of giving many treatments and decrease the clinical course.

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