Resistance mechanisms in lung cancer patients with EGFR or ALK aberrations treated with kinase inhibitors

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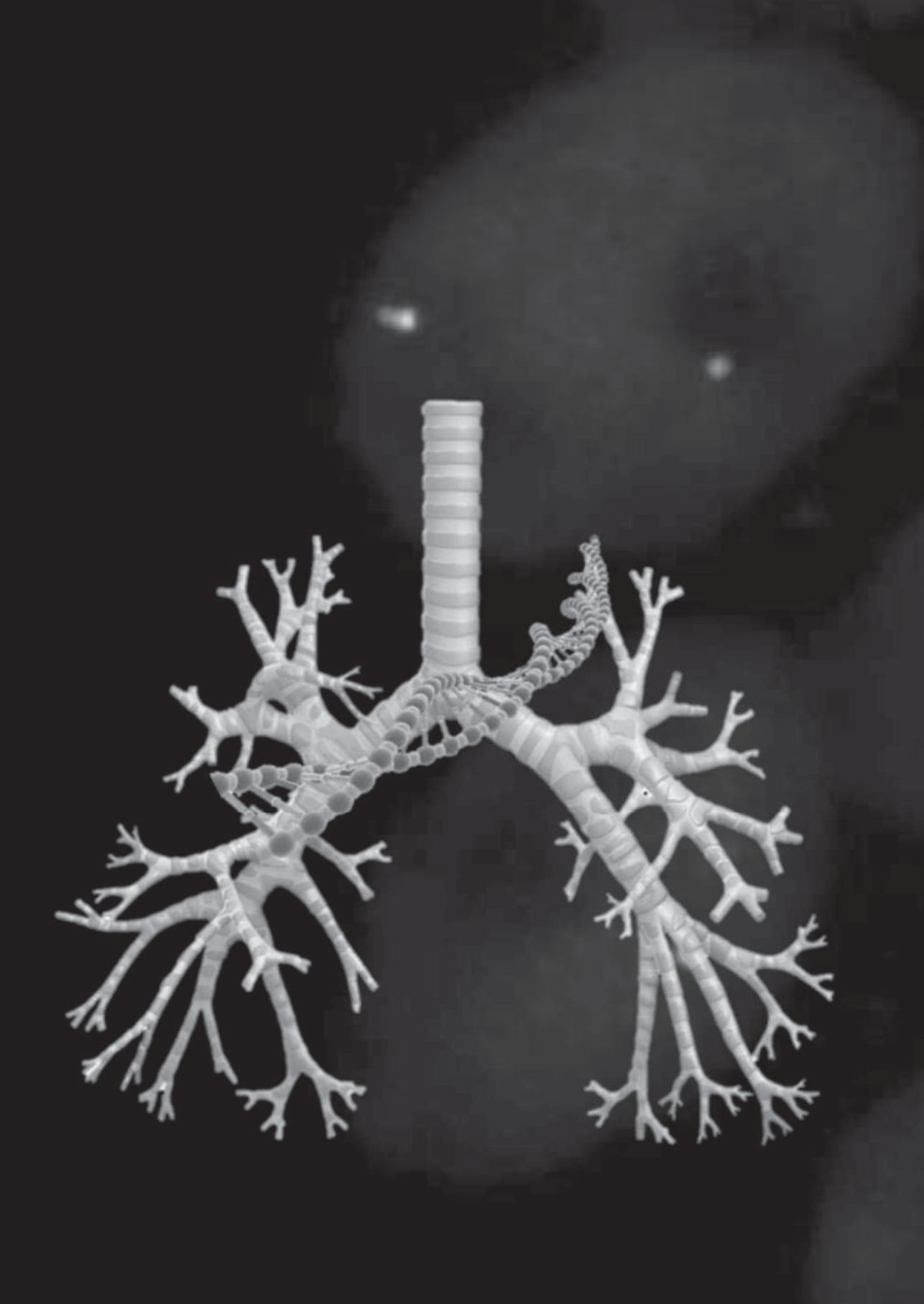
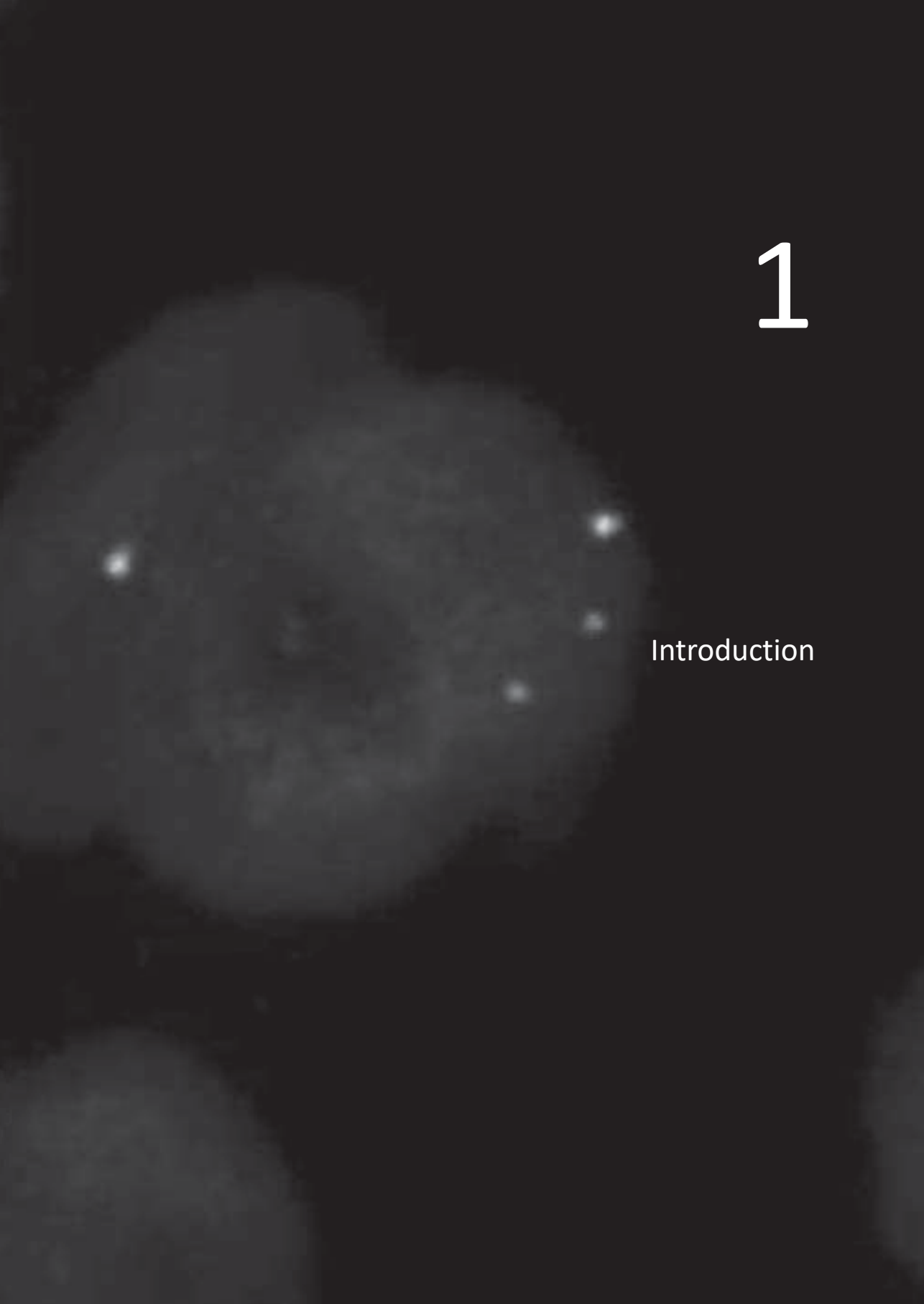
Contents





1

Introduction

1. General introduction

1.1. Lung cancer epidemiology

Non-small cell lung cancer (NSCLC) is the number one cause of death among cancer patients in the world[1,2]. The 10-year prevalence in the Netherlands was 23,726 patients in 2015. In this population, 55% of patients were male and 45% female. The incidence of lung cancer was 12,192 patients in 2015[3]. About 57% of patients had advanced (stage IV) disease. Among those patients around 40% have adenocarcinoma. Other subtypes are for example squamous cell carcinoma (25-30%) and large cell carcinoma (10-15%)[4]. About 80% of the lung cancers are induced by smoking and have a high burden of mutations (about 10.5/Mb) while the non-smoking induced cancers have a low mutation burden (about 0.6/Mb). This latter group often has specific DNA mutations – mostly single nucleotide mutations, deletions or rearrangements – in druggable driver genes[4,5]. For that reason many groups only analyse a limited number of genes in their diagnostic panels. In about half of the lung cancers, however, no druggable driver mutations have been identified (Figure 1.1)[6,7].

Kirsten Rat Sarcoma (*KRAS*) and Epidermal Growth Factor Receptor (*EGFR*) are the most frequently mutated genes in adenocarcinoma and multiple well-characterised activating mutations have been reported in these genes[8,9]. Genes activated by chromosomal rearrangements include the Anaplastic Lymphoma Kinase (*ALK*) gene[10]. In many cases, ALK activation is induced by a chromosomal inversion, resulting in an Echinoderm Microtubule-associated protein-Like 4 (EML4)-ALK fusion protein.

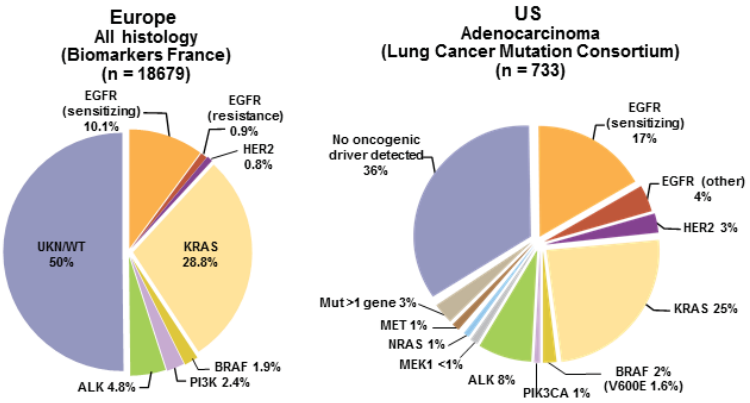


Figure 1.1. Mutations and translocations in lung cancer in European (all histologies) and US (adenocarcinoma) cohort (used with courtesy form F. Barlesi and adapted from Kris et al., JAMA 2014).

2. Genomic aberrations in lung cancer

2.1. Mutation analysis

Sequencing techniques have evolved from Sanger sequencing, to pyrosequencing and in more recent years to Next Generation Sequencing (NGS). Before NGS, until approximately 2013, high resolution Melting (HRM) analysis of PCR products has been used in combination with Sanger sequencing (Figure 1.2) to identify gene mutations to guide therapy decision making[11,12]. Since the start of targeted mutation screening in genes like *EGFR* and *KRAS* by Sanger sequencing, found in 1982 and 1993, the number of genes found to be mutated in lung cancer increased exponentially with the development of NGS platforms from manufacturers such as Illumina and Ion PGM™[13,14]. To date, a multitude of mutations have been identified and their relevance is currently being explored. At this moment, at the University Medical Centre Groningen, we use a panel of 25 clinically relevant predictive genes for routine diagnostic testing, in advanced stage lung carcinoma, melanoma, glioblastoma and colorectal carcinoma, to enable optimal therapy selection (www.moloncopath.nl; Table 1.1).

2.2. Chromosomal rearrangement analysis

Since the 1990s identification of chromosomal rearrangements has been performed using multiplex fluorescence in situ hybridization (M-FISH) and spectral karyotyping (SKY), whereas copy number aberrations were detected by FISH or comparative genomic hybridization (CGH)[15-17]. These techniques originally had a relatively low resolution of 5-10 Mb. With the development of array-based CGH, the resolution increased with a factor 10-100[18]. At this moment, FISH analysis is still commonly used to identify known

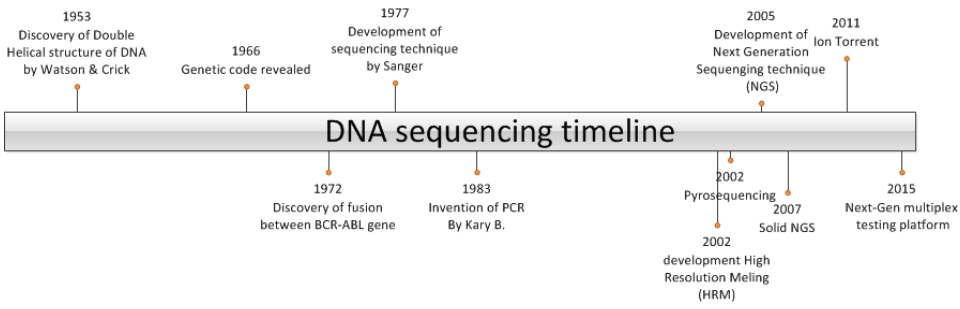


Figure 1.2. DNA analysis time line: An overview is given from the discovery of the DNA structure, to the development of PCR techniques and more recently the high throughput sequencing techniques.

Table 1.1. Overview of the IonTorrent-PGM gene panel currently used in the diagnostic setting at the Department of Pathology in the UMCG. (www.moloncopath.nl).

Gene name	Chromosome	Gene name	Chromosome	Gene name	Chromosome	Gene name	Chromosome
AKT	chr14	GNA11	chr19	IDH2	chr15	PDGFRA	chr4
ALK	chr2	GNAQ	chr9	JAK2	chr9	PIK3CA	chr3
AMELY	chrY	GNAS	chr20	KIT	chr4	POLE	chr12
BRAF	chr7	H3F3A	chr1	KRAS	chr12	ROS1	chr6
EGFR	chr7	H3F3B	chr17	MAP2K1	chr15		
ERBB2	chr17	HRAS	chr11	MET	chr7		
ESR1	chr6	IDH1	chr2	KRAS	chr1		

rearrangements in a diagnostic setting. For most of these rearrangements, dual-coloured fluorescent probes mapping upstream and downstream of the gene of interest are used to determine co-localization or segregation in formalin-fixed, paraffin-embedded (FFPE) tissue sections. For breaks in the *ALK* gene, FISH standards have been determined to provide a standardized clinical test (Figure 1.3)[19]. However, the interpretation of ALK-FISH patterns is challenging and time-consuming and subject to high observer differences[20]. Similar FISH standards are used for the detection of rearrangements in ROS1, RET and NTRK1. Alternative methods to detect activation of ALK and ROS are studied such as RNA-based RT-PCR approaches to detect expression of gene fusions or immunostaining to detect protein expression levels[21,22]. However, today reliable data are available only on the association of TKI-responses and rearrangements detected by FISH, whereas data for other fusion gene detections methods are lacking. To detect gene amplifications of for example the *MET* gene locus on chromosome 7, the FISH technique is also widely used in the clinical setting. For this analyses, a MET gene probe is combined with a chromosome 7 specific centromeric probe enabling to discriminate between normal patterns, gain/amplification of the MET gene and chromosome 7 polysomy[23,24]. Similar FISH approaches are developed for the detection of amplifications of FGFR1 and EGFR.

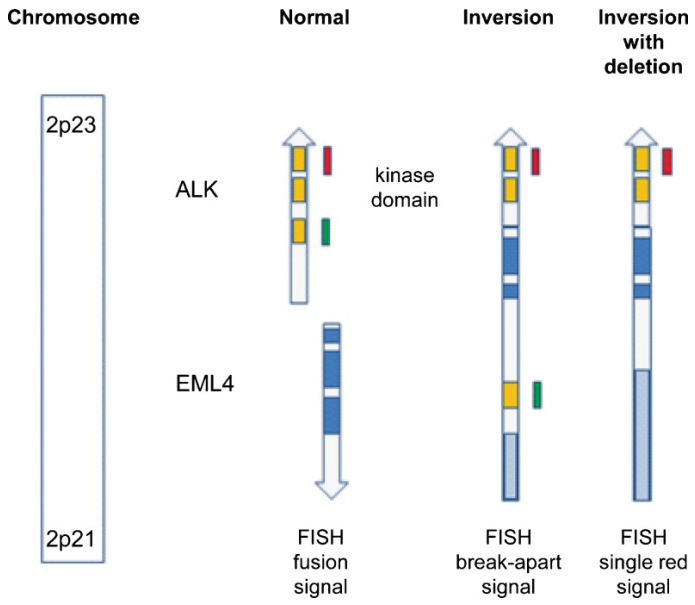


Figure 1.3. Probe design of the Vysis ALK Break Apart FISH Kit.
Segregation of the two signals or loss of the green signal, both indicate presence of a break in the ALK gene region. (Used with courtesy of E. Thunnissen; Virchows Arch. 2012).

3. Targeted therapy

Tyrosine kinase inhibitors (TKI) have been developed to inhibit cellular receptors that influence the proliferation rate. Most TKIs specifically bind to the kinase domain of a protein that is activated by a specific genomic aberration, in EGFR mostly encoded by a genomic deletion in a part of exon 19 or L858R point mutation in exon 21. Examples of EGFR inhibitors are gefitinib, erlotinib and afatinib. Eventually, all TKI-treated patients develop progressive disease and interestingly in ~50% of resistant or recurrent carcinomas, a specific second EGFR-mutation (T790M) is observed. The T790M mutation is a so-called gatekeeper mutation. This means that the binding site (i.e. gate) to the tyrosine kinase domain has been altered due to this mutation preventing binding of the initial TKI. Osimertinib is an example of an TKI specifically developed for targeting the C amino acid residue at position 797 in the kinase domain that binds into the ATP binding pocket without hindrance of the EGFR T790M mutation[25].

The development of kinase inhibitors for ALK translocations is going even more rapidly with crizotinib, ceritinib, alectinib, brigatinib, lorlatinib and others (Table 1.2). These TKIs are oral drugs with particular pharmacokinetic properties and different blood-brain penetration. Most treatment-naïve patients with advanced NSCLC with a ALK rearrangement showing more than 15% breaks are sensitive to crizotinib. Usually, no gatekeeper mutations are

Table 1.2. EGFR-TKI and ALK inhibitors with their most important targets and pharmacokinetic properties

Drugs	Targets	Daily dose (mg)	Frequency (daily)	T max (h)[a]	T ½el (h)[b]
Afatinib	EGFR, HER2, HER3, HER4	40	Once	2-5	37
Dacomitinib	EGFR, HER2, HER4	45	Once	6	15
Erlotinib	EGFR	150	Once	4	36
Gefitinib	EGFR	250	Once	3-7	41
Osimertinib	EGFR	80	Once	6	48
Alectinib	ALK, RET	600	Twice	4	33
Brigatinib	ALK, EGFR	90/180	Once	3/3	29/25
Ceritinib	ALK, ROS1, IFG1R	750	Once	4-6	31-41
Crizotinib	MET, ALK, ROS1	250	Twice	4-6	42
Lorlatinib	ALK, ROS1	100	Once	1	23

a: Time to maximum serum levels of the drug after intake, b: plasma elimination half time.

observed in these patients, but they will develop specific ALK gatekeeper mutations depending on the kind of drug exposure. Brain metastasis will be a major problem and the latest drug show high blood-brain penetration.

4. Outline of this thesis

In this thesis, we aimed to study the presence of genomic aberrations in lung cancer, especially those that can be targeted either by registered or experimentally used drugs. Drug resistance may develop through specific gatekeeper mutations that are present either as a minor tumour clone before drug exposure or that will develop during drug exposure. The specific aims of this thesis were to find resistance mechanisms towards afatinib treatment of EGFR-mutation positive patients and towards crizotinib treatment in ALK-positive patients. The following questions have been posed.

Part 1: What do we know about targeted therapy and resistance mechanisms to targeted therapies? In *Chapter two* we provide an overview of the genomic aberrations identified using sequencing techniques in lung cancer. Our interest and focus will be on genomic changes relevant to targeted therapy. In *Chapter three* we provide an overview of the currently known resistance mechanisms that develop during afatinib, an EGFR-TKI, and crizotinib, an ALK inhibitor, during exposure in cell lines and xenograft models, and in patients with advanced NSCLC.

Part 2: Which resistance mechanisms can be detected in patients with advanced NSCLC with activating EGFR mutations progressing during treatment with afatinib? In *Chapter four* we describe outcome of a cohort of patients that have been treated with afatinib in terms of tumour response rate, progression-free and overall survival. Whole exome sequencing has been used to explore the emergence of treatment-induced mutations in post-afatinib compared to pre-afatinib biopsies. Pathway analysis of recurrently mutated genes has been performed to identify novel resistance mechanisms.

Part 3: Which resistance mechanisms are found in patients with advanced NSCLC with ALK rearrangements progressing during crizotinib treatment? In *Chapter five* we explored the role of gene mutations in ALK-break positive advanced NSCLC patients by using whole exome sequencing on pre- and post-treatment samples. In *Chapter six* we explored crizotinib induced resistance mechanisms in ALK-break positive patients using RNA-seq on post-crizotinib tumour biopsies. The main aim was to identify the formation of new

fusion products associated with drug resistance. A significant percentage of ALK-FISH-positive patients treated with crizotinib do not respond well to crizotinib. In *Chapter seven* we studied clinical responses in a cohort of lung carcinomas with an ALK-rearrangement detected by FISH and treated with crizotinib comparing ALK-activation detected by FISH and ALK-expression levels. To determine ALK-expression we used a standardized, fast, simple dichotomized ALK immunohistochemistry test.

Part 4: Are protein prediction tests for EGFR better predictors for efficacy of targeted therapy? What to do with novel mutations in *EGFR*? In *Chapter eight* an overview will be given on the value of proteomics in predicting response to EGFR-TKI. In *Chapter nine* we present a patient with a c.2239_2240TT>CC: p.L747P *EGFR* mutation and the consequences for therapy.

Part 5: Conclusions

In *Chapter ten* we provide a general summary and will present an overall conclusion with a general discussion on *EGFR* mutations and *ALK* translocations and the targeted drugs that are used in different treatment lines.

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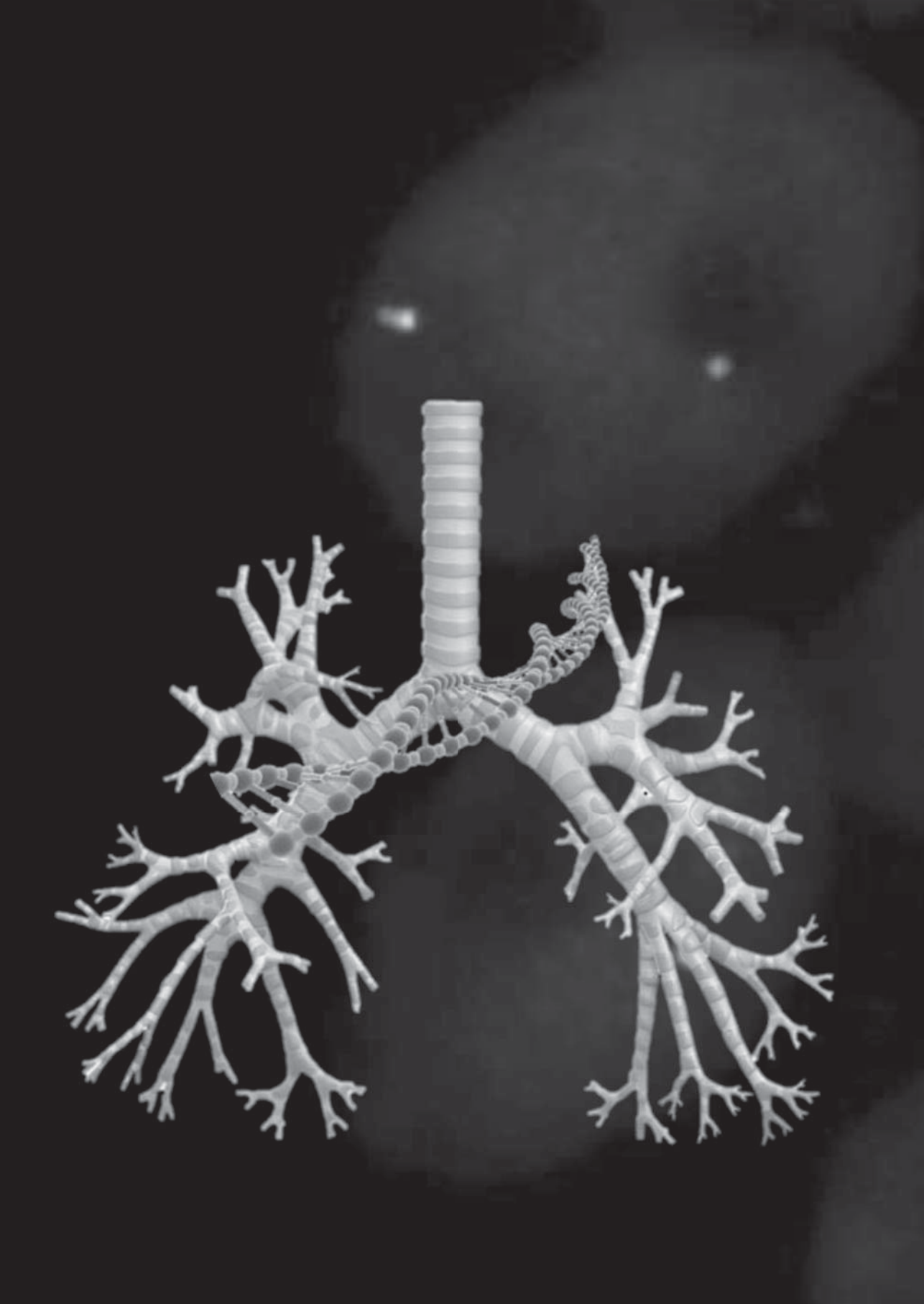

2

Genomic aberrations guiding treatment of non-small cell lung cancer patients

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Abstract

Lung cancer is the main cause of cancer-related death worldwide and conventional treatment strategies must be improved. In addition to mutations in several well-known cancer-associated genes, recent advances in sequencing technology have led to the discovery of numerous novel gene mutations and translocations. Some of these genomic aberrations occur at similar frequencies in all lung cancer subtypes, whereas others appear to be specific for adenocarcinoma or squamous cell lung cancer. High frequency mutations or recurrent translocations support involvement of the affected genes in the pathogenesis of lung cancer. The presence of activating aberrations is indicative for putative driver genes that might be essential for tumour cell growth and survival. These driver genes are potential targets for developing new treatments for lung cancer patients. Indeed, multiple tyrosine kinase inhibitors (TKIs) are currently used to treat lung cancer patients based on the presence of activating mutations, and novel drugs are under investigation. Patients benefit for about one year from current targeted treatments, but progression of disease inevitably occurs and resistance of the tumour to the TKI used can be observed in re-biopsied tumour samples. The aim of this review is to provide an overview of mutated genes in non-small cell lung cancer, an overview of targeted treatment strategies that are currently applied, and the known resistance mechanisms.

1. Lung cancer

Lung cancer is the leading cause of cancer-related deaths worldwide, with over 228,000 new cases and more than 159,000 deaths reported in 2013 in the United States[1,2]. Overall, the 5-year survival rate is about 16% and late diagnosis is significantly associated with poor prognosis[1]. Lung cancer can be divided into two main subtypes based on histology: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Approximately 85% of lung cancer patients are diagnosed with NSCLC[3], which can be further subdivided into three main groups, i.e. adenocarcinoma (AC), squamous cell carcinoma (SQCC), and large cell carcinoma[4]. The AC subtype used to be more frequent in women and non-smokers, but nowadays it is more frequent than other histological subtypes in both men and women[3]. The diagnosis of lung cancer is made by histology/cytology of a tumour detected by imaging techniques such as computed tomography (CT) and positron emission tomography (PET)[5]. Treatment of lung cancer patients depends primarily on the performance status, stage of the disease, the presence of oligometastases and on histological type[6]. Surgery is the primary treatment for patients with stage I or II NSCLC[7], although adjuvant chemotherapy is advised by many guidelines to increase survival of the patients. In non-resectable, stage III NSCLC disease, chemoradiation is the preferred treatment[8]. Nowadays, treatment of lung cancer patients with advanced disease is guided by mutation analysis in the case of a documented tumour-driver mutation. The number of different tyrosine kinase inhibitors (TKIs) available for the treatment of non-small cell lung cancer patients is rapidly increasing due to new diagnostic developments. In this review we give a brief overview of genes mutated in lung cancer, followed by a more in depth overview on potential therapeutic targets identified by next generation sequencing (NGS) technology. We also provide an overview of current targeted treatment approaches and the known resistance mechanisms.

2. Mutational landscape of lung cancer

Lung cancer, like other malignant neoplasms, is a result of an accumulation of different genetic alternations during life[9]. The *TP53* gene, originally described in 1979, was the first tumour suppressor gene to be discovered[10]. TP53 is mutated in approximately 45% of NSCLC patients[11]. In 1982, a human gene with transforming activity was identified in a lung carcinoma cell line. This gene is homologous to the Kirsten Rat Sarcoma virus and was referred to as *KRAS*[12]. Mutations in *KRAS* are mostly found in codons 12, 13, and 61. They occur more frequently in patients with AC (5-40%) than in other subtypes of lung cancer, and are associated with smoking behavior[9].

Developments in sequencing technologies in recent years and the need to identify novel therapeutic targets have encouraged scientists to sequence large numbers of lung cancer samples. Entire gene families like protein kinases or a combination of genes known to be mutated in lung cancer and other cancer types have been analysed[13-15]. Analysis of 518 protein kinases in 33 primary lung tumours and cell lines revealed 188 somatic mutations in 141 genes, including genes known to have a role in lung tumour genesis. For 21 genes, mutations were found in more than two samples. Seven of these genes had mutations in the kinase domain, including *BRAF*, *MAP2K4* and *FGFR2*. In addition, activating mutations were identified in *FGFR1* and *EPHA5* and inactivating mutations in *ATM*[13]. Analysis of 623 genes in 188 lung AC specimens by Ding et al. 2008 revealed 26 frequently mutated genes, including well-known cancer related genes such as *TP53*, *RB1*, *EGFR* and *KRAS*[15]. In addition, they also identified mutations in oncogenes such as *ERBB4* (*HER4*), *ERBB2* (*HER2*) and in multiple ephrin receptors (EPHAs). Altogether they observed a significant excess of mutations and copy number changes in genes involved in the mTOR, MAPK, Wnt, and the p53 signalling pathways[15]. Mutation analysis of the coding regions of more than 1,500 genes of 134 primary lung tumours revealed that 18 and 19 genes were mutated at a frequency significantly above the background mutation rate in AC and SQCC, respectively. Five of these genes including *TP53*, *KRAS*, *KEAP1*, *MUC16*, and *BAI3* were shared between AC and SQCC. Differences in the set of mutated genes for various subtypes suggest that different mechanisms are involved in tumour genesis[16]. Targeted sequencing of 145 cancer-related genes in 24 NSCLC biopsy samples, by Lipson and colleagues in 2012, revealed recurrent mutations in 21 genes, including well-known lung cancer genes together with mutations in druggable lung cancer genes such as *BRAF* and *EGFR*[17].

Together, these initial targeted and high throughput approaches indicated several targets, such as *EGFR*, *KRAS*, *BRAF* and *EML4-ALK*, that are nowadays treated with selected targeted drugs in the clinic. Although only a small proportion of all NSCLC patients (approximately 7%) will profit from these treatments (patients with complete and partial response), several tens of thousands of patients can still benefit worldwide because about 25% of patients with the subtype histology AC are suitable for studies with targeted therapies.

2.1. Potential therapeutic targets identified by next generation sequencing

Whole genome and exome sequencing (WGS and WES, respectively) have enabled researchers to dig even deeper into the mutational landscape of different cancer subtypes. These developments led to increased output of sequencing studies[18]. NGS gives us the

opportunity to generate large amount of sequencing data within limited time period in a more cost effective way compared to conventional sequencing. Although, NGS is being improved every day, still we need to be careful in data interpretation and mutation calling. For instance, artefacts that can occur during sample preparation, amplification bias should be taken into account while working with NGS data[19].

A comprehensive overview of mutation frequencies per gene for all types of cancer is given in the COSMIC database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/). For lung cancer, the top-20 most commonly mutated genes are shown in Figure 2.1. The first studies on lung cancer using massively parallel sequencing have been performed on either cell lines or single primary tumors[20-22]. A complete genomic analysis of a single NSCLC

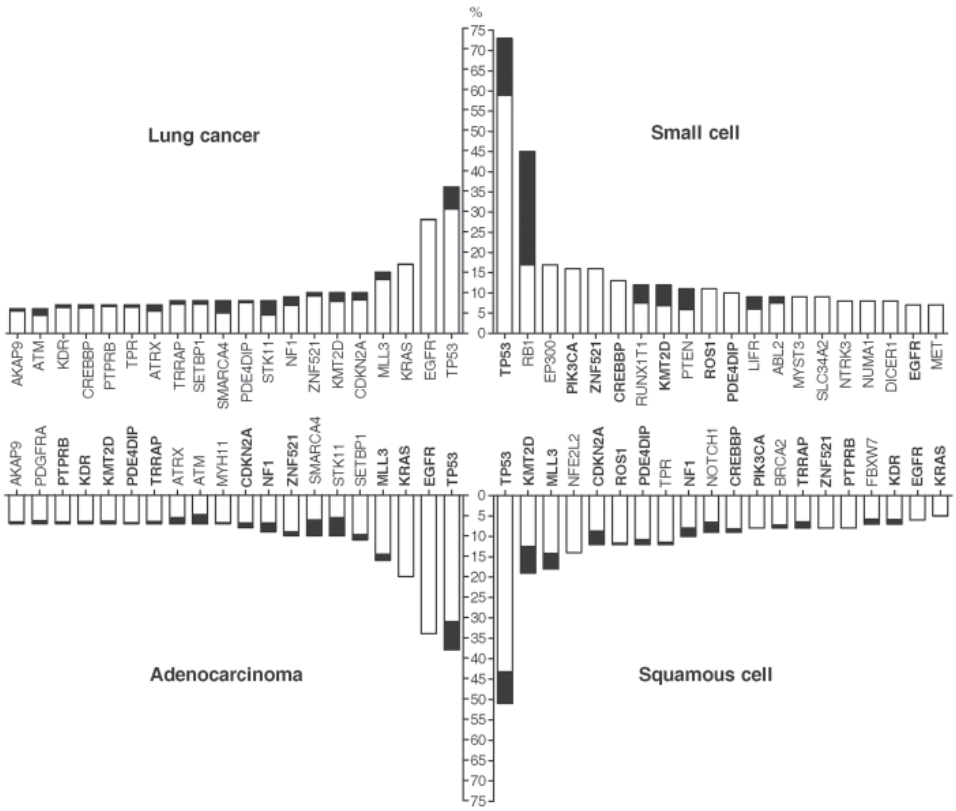

Figure 2.1. A schematic representation of the top-20 mutated genes in lung cancer overall and in the three main subtypes based on the COSMIC database.

In this database, mutation frequencies were calculated using a weighted average mutation frequency based on sample size across all studies. See the COSMIC website for more information (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/). Black bars: Nonsense and indel frameshifts; white bars: missense, synonymous, in-frame ins/del, complex and other mutations. Bold genes: These are among the top-20 genes in at least 2 out of 3 subtypes.

primary tumour revealed more than 50,000 somatic variations, including new mutations in genes known to be mutated at a high frequency in lung cancer, such as *NF1*[20]. Mutation analysis using WGS on a lung AC and a liver metastasis of the same patient resulted in the detection of a *KIF5B-RET* fusion gene similar to a previous report[17,23]. As *KIF5B-RET* is quite common, it might have an important functional role in the pathogenesis of lung cancer.

Liu and colleagues (2012) performed WES on a cohort of 31 primary non-small cell lung cancer (NSCLC) tumours and showed somatic mutations in several known lung cancer genes, including some of the targetable ones such as *EGFR* and *ERBB2* (*HER2*). Moreover, mutations were identified in *DCC* and *MML3*, which are targets for treatment in colon cancer and leukaemia, respectively. *CSMD3* mutations were detected in 19% of the patients; notably, this gene was the second most frequently mutated gene in their cohort after *TP53*[24].

WGS in a panel of 17 NSCLC primary tumours revealed nine commonly mutated genes, including several known lung cancer genes and three novel lung cancer genes such as *DACH1*, a tumour suppressor gene in prostate cancer and gliomas, and two fusion genes: *EML4-ALK* and *KDELR2-ROS1*[25]. They also detected mutations in *JAK2* similar to a previous report using a targeted approach[17]. JAK2 inhibitors are used to treat patients with myelofibrosis[26].

One of the largest studies to date performed WES on 178 lung SQCC samples and identified over 48,000 non-silent mutations[27]. As expected, *TP53* was the most commonly mutated gene with a frequency of 81%. Inactivating mutations in *CDKN2A*, *KEAP1*, *CUL3*, *NOTCH1* and *NOTCH2* were identified, as well as activating mutations in and amplification of *NFE2L2*, a key component of the oxidative stress response pathway[27]. Activation of the NFE2L2-KEAP1 signalling pathway through mutations in *NFE2L2* is a characteristic of paediatric liver tumours as well[28]. The Cancer Genome Atlas (TCGA) Network also found *SOX2* and *TP63* to be activated via amplification. These two genes are important components of the squamous cell differentiation pathway. They identified three potentially targetable tyrosine kinase families, including ERBB, FGFR and JAK, all of which were mutated and/or amplified[27].

In a study of 183 lung AC samples, 25 genes were significantly mutated, of which 19 had been reported previously and six were novel genes in lung AC, including *CHEK2* and *BRD3*[29]. In this study the authors also found inactivating mutations in *STK11*, *PTEN*, *RB1*, *SETD2* and *CDKN2A* and *CTNNB1* was found to be mutated in 3% of the patients. This gene is highly mutated (70%) in liver cancer patients and it is a component of WNT-CTNNB1 pathway[28,29].

A study on a large cohort of Korean patients (n = 104) with SQCC showed a good overlap with sequencing data previously published by TCGA Network, but also reported some marked

differences[27,30]. *CDKN2A* mutations were less common in Korean patients, while mutations in *RB1* were significantly more frequent. Interestingly, they found activating mutations in *PIK3CA*, which is one of the therapeutic targets of the PI3K/AKT/mTOR pathway. In one of the Korean lung cancer samples, an FGFR3-TACC3 fusion transcript was detected[30]. *FGFR3* has been reported as a potential therapeutic target in glioblastoma and bladder cancer[31,32]. Together, these next generation studies have made significant contributions to the identification of genes that are of interest as novel targets for therapy. To select the most promising target, it is essential to reveal the impact of the mutations and to discriminate between activating driver mutations and non-driver or inactivating mutations. In summary, next generation and targeted sequencing indicate that genes such as *ERBB2* (*HER2*), *ERBB4*, *JAK2*, *RET*, *ROS1*, *DCC*, *MLL3* might be good candidates for targeted treatment in lung cancer patients. In addition, inhibitors of the MEK kinase could be tested in tumours with *NF1* mutations. More importantly, *PIK3CA* seems to be a suitable candidate, together with *FGFR3* and *NFE2L2*, for treatment of patients with SQCC. At the moment, inhibitors for RET and ROS1 fusions are in preclinical and clinical trials and ROS1 inhibitors might also be effective on patients with activating *ROS1* mutations.

3. Targeted therapies currently in use

Specific aberrations in genes or pathways can lead to increased protein levels, and/or pre-active protein kinases that stimulate tumour cell growth. These aberrations can be targeted with small molecules such as TKIs (administered orally) and/or with monoclonal antibodies that are administered intravenously. At the moment, more clinical targets have been discovered in AC than in SQCC. Commonly used therapies in AC target the tyrosine kinase part of *EGFR*, *HER2*, *VEGFR* and ALK proteins[33]. In addition, a number of novel drugs against *KRAS* (AC/SQCC), *BRAF* (AC/SQCC), *ROS1* (AC), *RET* (AC), *FGFR1* (SQCC), *PIK3CA* (AC/SQCC) and *DDR2* (SQCC) are being evaluated in clinical trials or soon will be[34]. The known target genes, the kinase inhibitors used, and clinical outcomes in non-SQCC clinical trials phase III are summarized in Table 2.1. No phase III study has been performed in SQCC yet. Therefore, we only focus on non-SQCC. Frequencies of hotspot mutations, fusion genes and possible resistance mechanisms are summarized in Tables 2.2 and 2.3.

Table 2.1. Overview of targeted phase III studies in advanced non-SQCC

Protein/Inhibitors	Mutation % in non-SQCC subtype	Study	Progression-free survival hazard ratio (95% CI)
EGFR	10%		
erlotinib		EURTAC	0.37 (0.25-0.54)
		OPTIMAL	0.16 (0.11-0.26)
gefitinib		IPASS	0.48 (0.36-0.64)
		WJOTG3405	0.52 (0.38-0.72)
		NEJ002	0.32 (0.24-0.44)
afatinib		LuxLung 3	0.58 (0.43-0.78)
		LuxLung 6	0.28 (0.20-0.39)
BRAF	3%		
dabrafenib			N/A
trametinib			N/A
vemurafenib			N/A
ALK	3-6%		
crizotinib		PROFILE 1007	0.49 (0.37-0.64)
alectinib			N/A
ceritinib			N/A
ROS1	1%		
crizotinib			N/A
RET	1%		
vandetanib			N/A
sunitinib			N/A
sorafenib			N/A

N/A: Not available.

3.1. EGFR inhibitors

TKIs, such as erlotinib, gefitinib, and afatinib, are currently registered for treatment of *EGFR* mutant non-SQCC patients. Patients with activating *EGFR* mutations, such as exon 19 insertions/deletions, and nonsynonymous mutations such as G719X in exon 18, or L858R and L861Q in exon 21, are sensitive to erlotinib and gefitinib, with a tumour response rate of about 75%. Patients with such mutations show an increased progression-free survival (PFS) after targeted treatment compared to treatment with chemotherapy[35].

This effect was observed in both first and second line treatment. Hazard ratios for progression-free survival varied between 0.43 and 0.34. Most pivotal studies of first-line EGFR-TKI were limited to lung cancer patients carrying *EGFR* exon 19 deletions or L858R point mutations. Only patients with *EGFR* exon 19 deletions showed an overall survival

Table 2.2. Frequencies of mutation and fusion partners of therapeutic targets in non-SQCC

Target	Mutation	Fusion partner	Frequency	Reference
EGFR				91
Exon 18	G719C/S/A		7%	
	other		4%	
Exon 19	E746_A750del		37%	
Exon 20	V769M		2%	
	D770G/Y		5%	
	T790M		2%	
	other		5%	
Exon 21	L833F		5%	
	A840T		5%	
	L858R		26%	
	other		2%	
BRAF	V600E		50%	47
	G469A		39%	
	D594G		11%	
ALK		*EML4*	89%	34
		KIF5B	4%	
		KLC1	4%	
		TFG	3%	
		PTPN3	N/A	
ROS1		*SLC34A2*	40%	69,70
		CD74	40%	
		SDC4	20%	
		EZR	N/A	
RET		*KIF5B*	69%	77,78
		CCDC6	15%	
		NCOA4	8%	
		TRIM33	N/A	

benefit with afatinib. The effect of EGFR-TKI in patients with rare *EGFR* mutations has not been defined but seems less striking[36].

A meta-analysis revealed a significant association between increased *EGFR* copy number and improved survival outcome[37]. In NSCLC, *EGFR* mutation screening is the best method to predict tumour response to TKIs compared to fluorescence in situ hybridization (FISH) and immunohistochemistry[38]. In almost 25% of patients tumour growth is enhanced when *EGFR* treatment is ended, indicating that the tumour remains at least partly dependent on the EGFR signaling[39].

Table 2.3. Proposed resistance mechanism in targetable mutations and translocations in non-SQCC

Protein	Resistance mechanisms	References
EGFR	AXL/GAS6 axis activation	43
	Small cell transformation	41,42
	T790M mutation	40
	KRAS mutation	40
	MET/HER2 amplification	40
	Mutations in *NF-kB*/*ADAM17*/*NOTCH1*/*VIM*	43-45
	Mutations in COL6A1/ IGFR1/ Hedgehog/ TGFB/ p53/ Wnt/mTOR pathways	40,43
BRAF	NRAS/BRAF upregulation	54
	MEK mutation	54
	PTEN loss	54
	EGFR upregulation	52,54
	IGF-1R upregulation	54
	PDGFR-β upregulation	54
ALK	*ALK* mutations	15,63-65
	ALK copy number gain	63
	KRAS/*EGFR* mutations	65
	KIT amplification	92
	AXL overexpression	67
	EMT	67
ROS1	ROS1 mutations	73,75
	Signaling switch to EGFR	74
	KRAS/NRAS mutations	76
	KRAS overexpression	76

3.1.1. Mechanisms of resistance to EGFR inhibitors

After 9 to 12 months of treatment with an EGFR-TKI, the tumour appears to become resistant due to a spectrum of mechanisms. In about 50% of cases, an originally low-frequency TKI-resistant mutation, the T790M (gatekeeper) mutation, can increase to detectable and clinically relevant frequencies upon treatment with a TKI. In addition, amplification of *MET* or ERBB2, mutations in *PKI3CA* or *BRAF*, activation of the *AXL* kinase, and transformation to small cell lung cancer are the most prominently induced resistance mechanisms to TKI treatment[34,40]. These last two mechanisms have been proven in cell lines, xenografts or tumours of patients, and can therefore be considered as truly causative in relation to the resistance[41-43]. Some authors propose that new mutations developing in *EGFR* (like the T790M gatekeeper mutation) are associated with resistance in patients treated with EGFR-TKI, but also mutations in other genes such as *GAS6*, *VIM*, *NF-kB*, *ADAM17* and

NOTCH1 have been found in tumours of patients who became resistant to EGFR-TKI[40,43-45]. Finally, in studies using cell lines, mutations in *COL6A1*, *IGFR1*, *TGFB* or *mTOR* genes or mutations affecting the Hedgehog, p53, Wnt pathways were found to be associated with resistance mechanisms[43]. A true causal relation needs to be proven for these mechanisms.

3.2. BRAF inhibitors

BRAF is a serine/threonine kinase that is activated by RAS and signals its proliferative actions through MEK kinase. *BRAF* mutations are more common in lung ACs but are observed in less than 1% of NSCLC tumors[46]. In a study of 697 lung AC patients, *BRAF* mutations were present in 18 patients (3%), half were V600E (50%), the other most common mutations were G469A (39%) and D594G (11%)[47]. NSCLC patients with the *BRAF* V600E mutation have a shorter disease-free survival and overall survival after chemotherapy than patients without such mutations. At the moment, vemurafinib and dabrafenib are the two *BRAF* inhibitors that are clinically available. There are two case reports of lung AC patients with a V600E mutation who responded to treatment with vemurafinib[48,49]. The first, still ongoing, experience with dabrafenib in advanced NSCLC showed a tumour response rate of 40%[50]. Preclinical data suggest that non-V600E-mutated BRAF kinases are resistant to vemurafenib. In addition, *BRAF* mutations may predict sensitivity of NSCLC cells to MEK inhibitors[51].

3.2.1. Mechanisms of resistance to BRAF inhibitors

Different resistance mechanisms have been found in preclinical studies. A switch of full-length *BRAF* to aberrant *BRAF* (p61VE) has been shown in treated cell lines becoming resistant to BRAF inhibitors. Another mechanism that has been described in cell lines is upregulation of the EGFR protein due to loss of the *c-Jun* feedback loop[52]. A single lung cancer patient with a *BRAF* mutation and treated with dabrafenib showed a *KRAS* mutation in a re-biopsy at tumour progression. The hypothesis was therefore put forward that the cause of resistance was due to a G12D *KRAS* mutation[53]. Other mechanisms that have been described mostly in melanoma are MAPK-dependent mechanisms, like *NRAS* or *CRAF* upregulation, *BRAF* upregulation and *MEK* mutations. MAPK-independent mechanisms, like *PTEN* loss and upregulation of PDGFR-β and IGF-1R, have also been described[54].

3.3. ALK inhibitors

EML4-ALK is a fusion gene generated by an inversion of a segment of chromosome 2. It was the first targetable fusion onco-kinase identified in NSCLC[34]. It is most often observed

in young, light- or never smoking patients, occurring equally in males and females[55]. It can be detected in up to 4% of NSCLC patients and 3-6% of patients with lung AC[34,56]. *ALK* fusion genes rarely coexist with *KRAS* or *EGFR* mutations in lung cancer patients[57]. The EML4-ALK fusion protein results in enhanced ALK kinase activity[55]. Crizotinib was the first registered ALK inhibitor used in clinical practice (Table 2.1). It was originally designed for inhibition of the c-MET protein, but it turned out to have an inhibitory effect on ALK kinase as well[58]. The overall response rate in a FISH-based ALK-positive NSCLC group treated with crizotinib was 65% (95% confidence interval [CI]; 58-72) versus 20% (95% CI; 14-26) in the chemotherapy group. In a phase III study, the median PFS for crizotinib was 7.7 months. In the chemotherapy group PFS was 3.0 months[59]. Ceritinib and alectinib are also two potent second generation ALK-TKIs, that can be used in crizotinib resistant patients[60-62]. In a phase I clinical trial, Shaw and colleagues treated ALK-positive patients with ceritinib. A majority of these patients had been pre-treated with crizotinib. The overall response rate with ceritinib was 58% (95% CI; 48-67), median PFS was 7 months in those who received > 400 mg daily. Moreover, they observed tumour responses in patients with different *ALK* resistance mutations (L1196M, G1269A and S1206Y) as well as the patients without any detectable *ALK* mutation[61]. Treatment of an AC patient with alectinib (300 mg twice daily) resulted in complete response[62]. Both drugs are also effective for brain metastasis.

3.3.1. Mechanisms of resistance to ALK inhibitors

ALK-positive patients develop tumour resistance to targeted therapy. This resistance can be due to gatekeeper mutations in the kinase domain of the *ALK* gene (L1196M and G1269A), copy number gain of the EML4-ALK fusion gene, and mutations in *EGFR* and *KRAS*[63]. Other reported *ALK* mutations linked to resistance are V1135E, L1152R, C1156Y, F1174L, L1198P, G1202R, D1203N, S1206Y, G1269S, G1269A and L1318M. Targeted NGS showed an association between the development of resistance to crizotinib in two patients and new nonsynonymous mutations outside the ALK kinase domain[15,63-65]. The observation of different nonsynonymous mutations in *MET* could be important because crizotinib also is a potent MET inhibitor[66]. And lastly, mutations in pathways of *AXL* and the development of epithelial-mesenchymal transition (EMT) have been described as factors that contribute to ALK-TKI resistance[67].

3.4. ROS1 inhibitors

In about 1-2% of patients with NSCLC, a translocation of *ROS1*, which is located on chromosomal region 6q22, has been found with different fusion partners (*SLC34A2*, *SDC4*, *CD74*, *EZR*). Preclinical studies and case reports show that *ROS1* kinase activity is inhibited by crizotinib. *ROS1* fusions occur more often in younger patients and in the AC subtype[68,69]. Crizotinib is a potent inhibitor of cell growth in cell lines as well as in patients with a *ROS1* fusion[70]. A phase I clinical trial by Shaw and colleagues on fifty ROS1-positive patients with AC subtype treated with crizotinib showed an overall response rate of 72% (95% CI; 58-84) and the median PFS of 19.2 months[71]. Another study with 32 AC patients with ROS rearrangements treated with crizotinib revealed an overall response rate of 80% and median PFS was 9.1 months[72]. No randomized studies have been published yet.

3.4.1. Mechanisms of resistance to ROS1 inhibitors

A G2032R mutation of *ROS1* was found in a crizotinib-resistant patient. Foretinib (a MET and VEGFR inhibitor) seems to be a potent compound to overcome this resistance[73]. In the search for resistance mechanisms in cell lines, a switch in signalling from ROS1 to EGFR was observed, when ROS1 was inhibited by crizotinib[74]. The same study also reported that treatment with an EGFR inhibitor in combination with a ROS1 inhibitor was effective in cell lines resistant to ROS1 inhibitors. In another study, a new mutation, L2155S, was found in a ROS1-positive NSCLC cell line resistant to crizotinib. Authors also showed that L2155S and G2032R mutations can induce resistance to crizotinib in Ba/F3 cells[75]. KRAS/NRAS mutations or KRAS overexpression have been shown as other possible resistance mechanisms to crizotinib in HCC78-crizotinib resistant cell line[76].

3.5. RET inhibitors

A translocation of the rearranged during transfection (RET) gene located at chromosome 10 can be identified in about 1% of non-smoking patients with lung ACs. *KIF5B*, *CCDC6*, *TRIM33* and *NCOA4* serve as fusion partners[77,78]. As a result of this fusion, the normally low expression level of RET is increased in lung AC cells[79]. Over 12 drugs have been described wit RET inhibitory properties[80]. Most potent were cabozantinib (IC_{50} 4nM), alectinib (IC_{50} 4.8nM) and ponatinib (IC_{50} 7nM)[80,81]. Vandetanib, sunitinib, sorafenib and cabozantinib are all multikinase inhibitors, and the first three show *in vitro* activity against RET expressing NIH3T3 and Ba/F3 cell lines[17]. It has been shown that alectinib can inhibit cell growth by suppressing phosphorylated RET both in cell line and RET-positive mouse model[81]. A case

report confirmed the anti-tumour effects of vandetanib in a NSCLC patient[82]. In a phase II clinical trial, cabozantinib was tested on three RET-positive patients. Two of the patients showed a partial response, while the third patient had stable disease[78].

3.6. HER2 and cMET inhibitors

HER2 is mutated (mostly exon 20) in approximately 2% of NSCLC patients[83]. Activating mutations in *HER2* will result in activation of downstream signalling pathways (AKT and MEK) leading to cell proliferation and survival[84]. Blocking HER2 in *HER2*-mutated cell line resulted in cell cycle arrest and cell death[85]. Treatment of sixteen NSCLC patients with different combination of HER2 inhibitors (showed a median PFS of 5.1 months[83].

cMET is a tyrosine kinase receptor (TKR) which is coded by MET proto-oncogene and it is widely expressed by cells with epithelial-endothelial origin[86]. Different mechanisms can lead to aberrant activation of MET such as *MET* gene mutation or amplification and cMET/HGF overexpression[87]. Preclinical studies revealed inhibition of cell growth by crizotinib in MET dependent lung cancer cell lines, while tivantinib is independent of MET signalling and results is apoptosis[88]. A phase I clinical trial showed that tivantinib in combination with erlotinib was well tolerated in advanced NSCLC patients based on CYP2C19 genotype[89]. A phase II study showed a median PFS of 3.8 months in erlotinib plus tivantinib group compared to erlotinib or tivantinib plus placebo group[90]. Several clinical trials are still ongoing.

4. Conclusion

Nowadays, more and more somatic mutations and fusion genes are being identified using NGS approaches. The affected genes can be considered as potential targets for treatment of NSCLC. At the moment, we only have a few gene mutations and fusion genes that can be targeted with TKIs, although many other specific TKIs are under investigation. A striking and common feature is that tumour resistance develops after about one year of targeted treatment. Therefore, the search for different resistance mechanisms is important so that treatment regimens can be adapted at an early enough stage. At the moment, most resistance mechanisms are described on the basis of their associations with newly detected mutations observed at disease progression. We need to put more effort into functional studies to discover the role of mutations in new and known cancer genes and to define novel therapeutic drivers, which may even be genes with mutations at low frequencies. This

is important, because even a gene with a low mutation frequency can save a considerable number of patients with NSCLC.

In the future, we need to integrate the NGS results with epigenetic, transcriptome, copy number and proteomic analyses. This should preferably be done in primary tumours, metastases and the subsequent relapses with developing resistance to gain a good insight into the tumour cell evolution and to help design strategies to treat lung cancer patients optimally. Moreover, complete overviews of the mutational landscape of each patient's tumour will aid providing personalized therapy to patients and allow a timely switch to drugs that attack or work round resistance.

Combination therapies of different targeting drugs that are based on this mutational landscape will probably be more effective in prolonging the survival of patients and increasing their quality of live. Nowadays, the treatment of cancer patients should be based more on their genetic profiles and less on traditional organ- or cancer subtype-based strategies.

Three kinds of lung cancer patient groups may emerge in the future. In the first group are younger patients -mainly past, light or non-smokers- with limited somatic genomic instabilities that have one or two driver genomic aberration(s) that can be targeted with small molecules or combinations of these drugs. In the second group, smoking patients with SQCC usually have many somatic genomic alterations. These genomic changes might result in many abnormal peptides or proteins that can be recognized by the immune system and may induce an immune response. These patients will profit from immunotherapy. The third group still needs chemotherapy.

5. References

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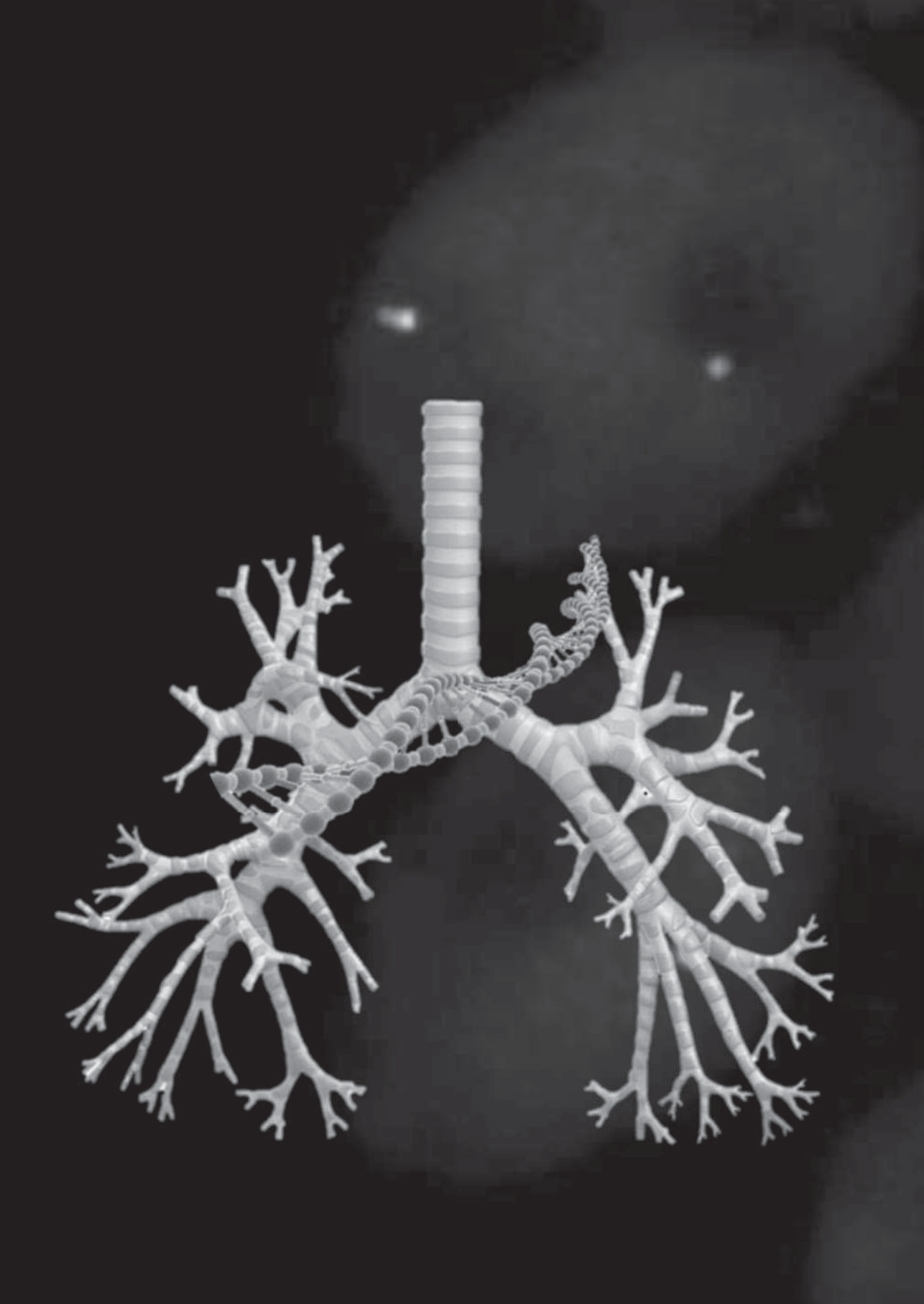

3

Resistance mechanisms after tyrosine kinase inhibitors afatinib and crizotinib in non-small cell lung cancer, a review of the literature

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Abstract

Targeted treatment of advanced non-small cell lung cancer patients with afatinib in *EGFR* mutation or crizotinib in *ALK* break positive patients results in profound tumour responses but inevitably induces resistance. In this review we present currently known resistance mechanisms for afatinib and crizotinib two recently approved drugs. Resistance mechanisms identified for afatinib include *c-MET* amplification and the V843I *EGFR* mutation. Expression of FGFR1, increased IL6R/JAK/STAT signalling, enhanced interference with aerobic glycolysis and autophagy are associated with resistance to afatinib.

Most common resistance mechanisms for *ALK* break positive cases are gatekeeper mutations in the *ALK* gene. Also activation of the EGFR pathway, *KRAS* mutations, the autophagy pathway and epithelial mesenchymal transition (EMT), have been associated with resistance. Many of the proposed resistance mechanisms need to be functionally studied to proof a causative relationship with resistance.

1. Introduction

Patients with an activating mutation in the epidermal growth factor receptor (*EGFR*) gene, e.g. deletion in exon 19 or L858R mutation, occur in about 8% of non-small cell lung cancer (NSCLC) patients[1]. In more than 70% of patients treatment is successful with EGFR-tyrosine kinase inhibitors (TKIs). However, resistance following treatment with TKIs eventually emerge in all of these patients. Most clinical data have been gathered with the first generation reversible EGFR inhibitors gefitinib and erlotinib (Table 3.1).

Table 3.1. Overview of drugs used as EGFR-TKI or ALKi and their most important targets

Drugs	Targets
Afatinib	EGFR, HER2, HER3, HER4
Dacomitinib	EGFR, HER2, HER4
Erlotinib	EGFR
Gefitinib	EGFR
Osimertinib	EGFR
Rociletinib	EGFR, IGF1R
Alectinib	ALK, RET
Brigatinib	ALK, EGFR
Ceritinib	ALK, ROS1, IFG1R
Crizotinib	MET, ALK, ROS1

Second-generation EGFR-TKIs, e.g. afatinib and dacomitinib, form covalent irreversible bonds with the target that may increase their effectiveness through a more effective inhibition of EGFR signalling. At this moment also 3rd generation TKIs are under investigation in phase III studies, e.g. osimertinib and rociletinib, for patients with an EGFR T790M mutation, because these TKI bind more specific to this altered tyrosine kinase binding pocket [2,3].

Around five per cent of NSCLC patients showed to have a chromosomal inversion involving the Echinoderm Microtubule-associated protein-Like 4 (*EML4*) gene and the Anaplastic Lymphoma Kinase (*ALK*) gene loci. These patients can be treated with ALK inhibitors (ALKi), e.g. crizotinib and ceritinib (Table 3.1)[4]. At this moment alectinib has only been approved in Japan. Ceritinib and alectinib bind more specific with the tyrosine kinase pocket of the

ALK protein due to covalent binding and the fact that these molecules are smaller than crizotinib. The median time to progression is 8 to 12 months for all TKIs when administered as first-line treatment, which is better than chemotherapy[5-8]. The median progression free survival (PFS) is comparable to first line treatment when these TKIs are used as second-line treatment following chemotherapy[9]. In subsequent treatment lines, however, survival is not different from chemotherapy.

Many genetic aberrations have been described as possible mechanisms for resistance towards TKIs[10]. In this review we will focus on resistance mechanisms, found in tumour biopsies of NSCLC patients, xenograft models and cell lines that emerge after treatment with or exposure to EGFR-TKI afatinib (Boehringer Ingelheim GmbH, Germany) and the ALKi crizotinib (Pfizer Inc., City of New York, NY). Both afatinib and crizotinib have only recently been approved for treatment and knowledge on known and possible resistance mechanisms is essential for clinicians.

2. Resistance after EGFR tyrosine kinase inhibitors

Afatinib, erlotinib and gefitinib are registered as first line treatment for patients with advanced NSCLC with activating EGFR mutations. Afatinib is the only proven drug showing an increased overall survival as first line treatment in patients with an exon 19 deletion as compared to chemotherapy, i.e. 31.7 (95% confidence interval (CI) of 28.1-35.1) vs. 20.7 (95% CI of 16.3-25.6) months, respectively[11,12]. However, for patients with a L858R mutation, the OS is comparable between afatinib and chemotherapy treatment (OS 22.1 months for afatinib; 95% CI of 19.6-25.4 vs. 26.9 months for chemotherapy; 95% CI of 23.2-31.7)[12]. An overview of currently known resistance mechanisms is given below. It consists of a summary of findings with first generation TKIs followed by a more extensive overview of resistance mechanisms associated with afatinib only.

2.1. Erlotinib and gefitinib resistance in NSCLC patients, cell lines and mouse models

The T790M mutation is the most common observed resistance mechanism (30-83%) in patients treated with first generation EGFR-TKIs[13-18]. The resistant tumour clones may originate from a minor pre-existing T790M mutation positive tumour cell sub clone or present as a *de novo* mutation[19]. Mutation screening of >2,700 lung pre-treatment cancer samples with an EGFR mutation revealed a T790M mutation in only 20 cases. This may indicate that without TKI selective pressure the frequency of the T790M is very low[20]. The T790M

mutation has also been described as a germ line variant co-segregating in a family with the development of lung adenocarcinoma[21,22]. A similar co-segregation with lung cancer was seen in a family carrying a germ line V843I and a family with a P848L *EGFR* mutation[23-25]. It is therefore most likely that resistant mutations are already present in minor clones of the tumour. In a clinical subgroup analysis of *EGFR* mutant patients in the EURTAC trial a pre-treatment T790M mutation was associated with a decrease in PFS. Bcl-2 interacting mediator of cell death (BCL2L11, also known as BIM) mRNA expression was associated with longer PFS and OS in the same patients treated with erlotinib[26].

Mice with lung epithelial cell specific overexpression of activated EGFR developed lung tumours. Upon treatment with erlotinib, T790M mutations were found in 5/17 and *MET* gene amplification in 5/17 different mice[27]. This indicated that both T790M and *MET* gene amplifications are potential resistance mechanisms to erlotinib.

HER2 amplifications and mutations were observed in lung cancer biopsies in respectively 10% and 2% of tumours with acquired resistance to erlotinib and gefitinib, but only in 1% of untreated tumors[28]. Therefore, *HER2* may be responsible for resistance emerging under pressure of treatment especially with erlotinib.

Phosphorylation of Src family kinase (SFK) was identified in tumour samples of patients treated with first generation TKIs[29]. Additional resistance mechanisms such as expression of HGF, the ligand for MET or increased activation of the AXL pathway, up-regulation of NF-kB, GAS6, ADAM17, NOTCH1, p53, Wnt and mTor were reported in studies focusing on tumour samples and cell lines[10,30-35].

2.2. Afatinib resistance in NSCLC patients

In Asian patients the presence of T790M mutations in re-biopsies obtained before second line treatment with afatinib treatment had no prognostic or predictive role[36]. This observation was confirmed in a study where a combination of afatinib and cetuximab (a monoclonal antibody against EGFR) showed tumour responses irrespective of the presence of T790M (Table 3.2)[37]. Thus questioning as to whether a T790M mutation is important as a resistance mechanism upon treatment with afatinib. Afatinib is equally potent against wild-type EGFR and EGFR T790M and therefore side effects resulting from inhibiting wild-type EGFR (rash and diarrhoea) prevents the suppression of T790M tumour cells at clinically achievable doses. In addition, patients treated with afatinib have more side effects, e.g. diarrhoea and skin rash, than observed with erlotinib or gefitinib treated patients[38]. Third generation EGFR TKIs such as rociletinib, osimertinib, or EGF816, target

mutant EGFR T790M and largely spares wild type EGFR, thereby decreasing toxicity and therefore permit clinical doses that fully suppress T790M. This explains their efficacy in T790M positive tumours.

Table 3.2. Overview of re-biopsy studies in patients treated with afatinib

Re-biopsy study	N	Mutation analysis	Treatment	Mutations	PFS (mo)	Reference
Sun et al. 2013	70	*Direct sequencing and sequencing using a peptide nucleic acid*	Afatinib	T790M+ T790M-	14.7 14.1	36
Janjigian et al. 2014	126	*NA / different methods*	Afatinib/ cetuximab	T790M+ T790M-	4.6 4.8	37

2.3. Afatinib resistance in cell lines, xenografts and mouse models

2.3.1. Gatekeeper mutations

Multiple cell lines have been used in the search for responsiveness and resistance mechanisms to afatinib. A summary of the endogenous and exogenous alterations in relation to TKI responsiveness is given in Table 3.3.

Cell lines carrying the activating exon 19 delE746_A750 or L858R mutations are sensitive to afatinib[23]. PLA1 cells with the activating L858R in combination with the resistant V843I mutation are more resistant to afatinib than H1975 cells with a L858R in combination with the T790M resistant mutation. This difference in sensitivity can be explained by conformational change of the predicted TKI binding site as a consequence of the V843I and the T790M mutations[23]. Both *EGFR* mutations are referred to as gatekeeper mutations, of which V843I appears to be a more resistant variant than T790M. In another study afatinib inhibits the growth of the H1975 cell line (containing the L858R / T790M mutations) in vitro as well as in the xenograft model[39]. In comparison to EGFR-TKIs, EGFR siRNAs were much more effective in growth inhibition of lung cancer cells[40]. This enhanced efficiency occurred especially in T790M mutation positive lung cancer cell lines[41]. This indicates that treatment with EGFR-TKIs only partly block the EGFR signalling pathway, in which especially T790M seems to be important as a resistance mechanism in cell lines.

Resistance upon exposure to afatinib in PC9 cells seems to be irreversible indicating that the resistance has been caused by a genetic change[42]. The majority of the resistant subclones remained dependent on EGFR signalling as shown by siRNA treatment. These EGFR dependent

Table 3.3. Overview of IC_{50} values in cell lines used in research on EGFR-TKI resistance

Cell line	Endogenous EGFR / induced alterations		Erlotinib (uM)	Gefitinib (uM)	Afatinib (uM)	Dacomitinib (uM)	Crizotinib (METi) (uM)	Ref
	EGFR	other						
A549	wt		>100	>100	0.01	0.003	646	23,45
H292	wt		>10	0.06				41
H358	wt	KRAS codon 12	25	>10				14,41
H1299	wt						642	45
EBC1	wt	MET amp					5	45
H596	wt	MET exon 14del					752	45
H1437	wt	MET exon 14del					1284	45
H1993	wt	MET amp					10	45
H2122	wt	MET N375S					472	45
HCC827	exon 19del		<0.1	<0.1	0.01		767	14,40,41,45
H1650	exon 19del		10-100	10-100	1-10			14,40
PC9	exon 19del		0.03	0.02	<0.01		787	44,45
PC9-GR	EGFR T790M ↑		3441	100,000	312			29,42,52, 53
PC9-BR		pHER2 ↓ pHER3 ↓ pFGFR1 ↑			0.02			48
H3255	L858R		0.04	0.04	0.0007			44
H1975	L858R / T790M		100	100	1	10		14,23,40,44
H1975-BR		EMT			29			52
PLA1	L858R / V842I		>100	>100	1-10	>10		23

wt: wild type; amp: amplification; GR: gefitinib resistant; BR: afatinib (BIBW2992) resistant.

PC9 clones all gained a T790M mutation and cell viability was dependent of the T790M allele dosage[42]. However, in the xenograft model no difference in tumour response was observed at maximum dose for afatinib between different T790M allele dosages. Other known resistance mechanisms such as amplification of *MET*, deletion of phosphatase and tensin homolog (*PTEN*) and epithelial-mesenchymal transition (EMT), were not observed in the afatinib resistant, EGFR independent PC9 subclones. In a mouse model, overexpression of L858R mutant *EGFR* in epithelial lung cells resulted in the development of lung cancer[27]. Upon treatment of these mice with erlotinib, resistant tumour clones gained a T790M mutation, which showed a modest response to afatinib monotherapy. Combining afatinib with cetuximab greatly enhanced the response with complete responses in most cases (7/8)[43]. This indicates that adding cetuximab to afatinib induces a higher response rate in T790M positive tumour cells. Another study suggested that this effect was related to the heterodimerization of tyrosine-phosphorylated EGFR with HER2, to which afatinib is a known inhibitor as well[44]. The differences observed between the cell line and mouse models in comparison to the above-discussed findings in patient samples are caused by differences in clinical achievable afatinib doses. Such afatinib doses are too low to suppress T790M mutated tumours.

2.3.2. *HER2* gene expression and receptor involvement in resistance

Knockdown of *HER2* in PC9, HCC827 and H3255 cells increased sensitivity to afatinib[44]. This is consistent with the improved response rate observed in patients treated with afatinib and cetuximab as compared to erlotinib and cetuximab or afatinib alone. Erlotinib-resistant PC9 and HCC827 derived xenograft tumours are characterized by enhanced levels of phosphorylated HER2. Proliferation of these HER2 positive tumour cells in nude mice could be inhibited by afatinib or cetuximab, with the strongest inhibition of HER2 being observed for treatment with afatinib/cetuximab[44]. Thus *HER2* is a mechanism of resistance induced upon treatment with erlotinib and gefitinib. However, this resistance mechanism is not relevant for afatinib, a known pan-HER inhibitor.

2.3.3. *MET* gene expression and receptor involvement in resistance

Amplification and/or mutations of *MET* have been identified in two of the lung cancer cell lines and may represent a possible escape mechanism to afatinib. Treatment of lung cancer cell lines harbouring activating *EGFR* mutations with crizotinib (a known MET and ALK inhibitor) showed a marked inhibitory effect on cell growth of *MET* amplification positive cell lines, indicating that these cells were addicted to the MET pathway. The

MET addiction was confirmed by increased levels of apoptosis upon *MET* inhibition in *MET* amplification positive cell lines, EBC-1 and H1993[41,45]. *EGFR* mutation positive cell lines with mutant or wild type *MET* were not or less sensitive to crizotinib and/or MET siRNAs, indicating that *MET* mutations are not important in resistance in these cells[41,45]. Combination of *MET* siRNAs with either EGFR-TKIs, including afatinib, or *EGFR* siRNAs, revealed decreased proliferation as compared to *EGFR* siRNA alone[41]. In the T790M positive H1975 cell line, the synergistic effect was modest, also when the *MET* siRNA was combined with a siRNA directed specifically against T790M. Concomitant treatment of the cell lines with a MET inhibitor (SU11274) caused increased sensitivity for gefitinib, erlotinib, afatinib or cetuximab, especially for the H1975 cells. The combined effect was most pronounced for the combination of SU11274 and afatinib[14]. In mice models, *EGFR* knock down in *MET* amplification positive tumours increased tumour cell death upon treatment with MET inhibitors. Mutations in *MET* did not affect sensitivity in mice models[14,41,45,46]. Thus, none of the studies support an effect of mutations or deletions of the *MET* gene towards TKI resistance, whereas amplification of *MET* does confer resistance to afatinib.

2.3.4. *STAT3* gene expression and receptor involvement in resistance

Two cell lines carrying the T790M *EGFR* mutation were used to explore the role of STAT3 activation in the TKI-treatment induced resistance cells[47]. Afatinib treatment induced autocrine signalling of the JAK/STAT pathway via secretion of IL-6 in H1975 and PC9-gefitinib resistant (GR) cells. Blocking of the IL-6 receptor pathway (IL-6R) combined with afatinib treatment showed a more pronounced growth inhibition than afatinib alone. Vice versa, activation of the IL-6R signalling decreased sensitivity to afatinib. Moreover, an acquired resistance to afatinib was observed in PC9-GR xenograft mice that showed increased STAT3 protein levels[47]. This indicates that upregulation of the STAT3 pathway may contribute to afatinib resistance.

2.3.5. *FGFR1*

The PC9-BIBW9292 (afatinib) resistant cell line (BR), showed decreased mRNA expression levels of *HER2* and *HER3* compared to the parental PC9 cells, whereas mRNA levels of *FGFR1* and its ligand FGF2 were increased. Combining afatinib treatment with *FGFR1*-siRNAs or FGFR1-TKI inhibited cell growth of PC9-BR cells and induced a marked decrease in phosphorylation of AKT and ERK phosphorylation[48]. Thus, this afatinib resistant cell line was addicted to FGFR1-induced survival signals.

2.3.6. SRC family kinase (SFK)

In a tyrosine phospho-proteomic study in PC9-GR cells expression of many TKs was enhanced as compared to the wild type cells[29]. Another study on SFK used a combination of dasatinib, a synthetic small molecule inhibitor of SRC-family protein-tyrosine kinases, and afatinib. This combination overcame the T790M mediated resistance of PC9-GR cells (IC50 of 36uM as compared to 312uM for afatinib alone)[31]. This result was confirmed in a xenograft model of nude mice injected with PC9-GR cells. These data indicate that SFK may present another mechanism to EGFR-TKI resistance.

2.3.7. Heat shock protein 90 (HSP90)

There are no studies in which the effect of HSP90 inhibitors has been tested on afatinib resistant cell lines. However, in cell lines resistant to erlotinib the combination of afatinib and the HSP90 inhibitor ganetespib induced destabilization of EGFR, MET, p-STAT3 and p-AKT proteins gaining more cell death than afatinib or ganetespib alone[49]. Ganetespib also induced a clear effect in CL-387,785 (pan-HER inhibitor) resistant clones or clones with an *ERBB2* exon 20 mutation[50,51]. This means that although not studied for afatinib yet, HSP90 could play a role in treating resistance to a pan-HER inhibitor.

2.3.8. Autophagy

Combined treatment of T790M positive PC9 and H1975 cells with afatinib and suberoylanilide hydroxamic acid (SAHA), a potent reversible histone acetyltransferases and histone deacetylases (HDAC) inhibitor, could overcome the EGFR-TKI resistance through activation of the apoptotic pathway. The enhanced apoptosis was shown to be dependent on the autophagy pathway[52]. These results were supported by *in vivo* data in xenograft mouse models.

2.3.9. Upregulation of other pathways

A genome wide screen at copy number, gene expression and protein levels in parental and afatinib resistant sub clones revealed amongst others activation of AKT in H1975 cells[53]. As activation of AKT has been associated with epithelial-mesenchymal transition (EMT) in prostate and breast cancer, EMT characteristics were also studied in the H1975 resistant sub clones[54,55]. This revealed loss of E-cadherin, decrease in cytokeratin levels, and increased expression level of mesenchymal markers such as vimentin. Based on these findings EMT was proposed as a possible mechanism of resistance[53].

Another mode of action of inhibition of AKT may be an effect on the glycolytic pathway. Inhibition of the glycolysis by 2-deoxy-D-glucose (2DG) in H1975 and PC9-GR cells potentiated the sensitivity to afatinib. Two modes of action have been proposed. First, afatinib inhibits the PI3K/AKT pathway and prevents AKT from switching off the glycolytic pathway providing growth-enhancing signals to the tumour cells. Alternatively, the emergence of resistance towards afatinib lowers the AKT inhibition and switches on the glycolytic pathway[56,57]. Both mechanisms depend on the 2DG pathway, which explains the increased sensitivity towards afatinib upon inhibition of the 2DG pathway.

Whether NF-kB, GAS6, ADAM17, NOTCH1, Wnt, mTOR and p53, found to be associated with resistance to first generation TKIs, play a role in resistance to afatinib is not obvious, as these mechanisms have not been studied yet[10,30-34].

3. Resistance after ALK tyrosine kinase inhibitors

Crizotinib is used as treatment in *ALK* translocation-positive patients. Besides ALK, crizotinib also targets MET and ROS1, although it has not been registered for these aberrations yet. Tumours of patients treated with crizotinib become insensitive to this drug after a median of 12 months (range: 1-34)[7,8]. An overview of the currently proposed and known mechanisms to crizotinib based on patient samples and on *in vitro* data from cell line studies is discussed below.

3.1. ALK inhibitor resistance in NSCLC patients

Analysis of crizotinib resistant tumour clones demonstrated new *ALK* gene mutations in 17 out of 53 patients, e.g. 1151Tins, L1152R, C1156Y, L1196M, S1206Y, G1269A (Table 3.4)[58-64]. Most of these mutations affected the P-loop, β-sheet or α-helix of the ALK protein in the so-called gatekeeper area. Based on the crystal structure of wild type and mutant (L1152R, G1202R, S1206Y and C1156Y) ALK protein, it was predicted that mutations close to the gatekeeper area resulted in a decreased interaction of crizotinib with the ALK receptor. *In vivo* experiments confirmed the accurateness of these predictions by increased resistance to crizotinib[65,66]. Thus these gatekeeper mutations prevent binding of crizotinib to the kinase domain of the ALK protein and due to this the binding of crizotinib becomes ineffective.

In addition to these gatekeeper mutations, *ALK* copy number gain has been found in almost 8% of resistant patients and *EGFR* or *KRAS* mutations have also been observed in almost 8% of the resistant tumour clones[60,61,64,67]. Unusually, *EGFR* activating mutations

Table 3.4. Overview of re-biopsy studies in patients treated with crizotinib

Re-biopsy study	N	Mechanism	Mutations	Technique	Ref
Sasaki et al. 2011	1	*ALK* mutation	L1152R	DNA Sanger sequencing	58
Sang et al. 2013	1	*ALK* mutation	G1269A	DNA Sanger sequencing	59
Katayama et al. 2012	4/18	*ALK* mutation		DNA Sanger sequencing	60
	1/18	*ALK* gain		FISH	
	4/18	Higher levels of phospho-*EGFR*		IHC	
	2/18	*KIT* amplification		Snapshot multiplexed genotyping assay, FISH	
	1/18	Overexpression of *KIT* ligand stem cell factor (SCF)		IHC	
Kim et al. 2013	3/7	*ALK* mutation	L1196M, G1269A	DNA Sanger sequencing	61
	1/7	*ALK* gain		FISH	
	1/7	*EGFR* activating mutation	L858R	DNA Sanger sequencing	
	1/7	*EGFR* polysomy		FISH	
	2/7	Overexpression of amphiregulin or *EGF*		ELISA	
Huang et al. 2013	3/13	*ALK* mutation	G1269A, C1156Y, L1196M	DNA Ion Torrent sequencing	62
Choi et al. 2010	1		C1156Y, L1196M	RNA paired-end sequencing of ALK kinase domain	63
Doebele et al. 2012	4/11	*ALK* mutation	L1196M, G1269A	DNA direct sequencing	64
	2/11	*ALK* gain		FISH	
	2/11	Loss of *ALK*		FISH	
	1/11	*EGFR* activating mutation	L858R	DNA direct sequencing	
	2/11	*KRAS* mutation	G12C, G12V	DNA direct sequencing	
Jiang et al. 2013	1	*ALK* mutation	G1548E	Illumina Hiseq 2000	67

were observed in 3/50 EML4-ALK positive, treatment naïve patient samples in only one study. This indicates that *ALK* breaks may co-exist with *EGFR* mutations[58]. However, in large studies ALK rearrangements and EGFR activating mutations were mutual exclusive. In another study, amplification of *KIT* and increased auto phosphorylation of EGFR was observed in 2/18 re-biopsies[60]. In some patients combinations of multiple resistance mechanisms were observed in the same tissue re-biopsy[68]. The data of these studies led to the concept that tumour cells can apply both ALK dependent and independent mechanisms to become resistant to crizotinib. In *ALK* gatekeeper mutant cases, the tumour cells remain addicted to ALK signalling. In contrast, ALK independent mechanisms refer to cases that have become addicted to an alternative oncogene[64].

In clinical settings the majority of resistant mechanisms appear to be ALK dependent, since responses are seen in up to 70% of crizotinib resistant tumours on second line ALK inhibitors like ceritinib, alectinib and brigatinib (Table 3.1). These drugs have a higher potency thereby avoiding the possibility of bypassing the ALK signalling cascade via other pathways[69].

3.2. ALK inhibitor resistance in cell lines and xenograft mice models

3.2.1. Gatekeeper mutations

Resistance to crizotinib has been studied in several cell lines (Table 3.5). The H838 and H23 cell lines carrying wild type *ALK* gene alleles, have been used as a control for the ALK break-positive cell lines H3122, Ba/F3 and H2228[70]. The SNU-2535 cell line carries besides an ALK break also a mutated *ALK* allele, i.e. G1269A[61]. The DFCI076 cell line gained a L1152R mutation[58]. *ALK* gatekeeper mutations have been described as the major resistance mechanism in different cell lines treated with crizotinib. Resistant H3122 cells (H3122-CR) mainly gained C1156Y, F1174L, L1196M and G1269A gatekeeper mutations[58-62,71,72].

The G1269A gatekeeper mutation carried by SNU-2535 and H2228 cells indeed showed increased resistance to crizotinib[61]. The L1152R mutation present in the patient derived DFCI076 cell line also decreased sensitivity to crizotinib[58]. Ba/F3 cells containing one of the three most effective resistance inducing gatekeeper mutations, i.e. L1196M, S1206R or G1269S, were insensitive to crizotinib in a xenograft model of SCID mice[70]. Introduction of the T1151K, L1152V, L1152R, C1156Y, I1171T, S1206R, E1210K, F1245C or G1269S gatekeeper mutations in the Ba/F3 cell line by site-directed mutagenesis, increased resistance to crizotinib by tenfold in each of the Ba/F3 subclones[58,63,64,70]. So there is solid evidence that *ALK* gatekeeper mutations are involved in resistance towards crizotinib.

Table 3.5. Overview of IC_{50} in cell lines used in research on crizotinib resistance

Cell line	Alterations		Crizotinib (uM)	Ref
	ALK	Other		
H23	wt		1.7	70
H838	wt		1.3	70
H2228	ALK-EML4		0.01	70,71
H2228-CR		EGFR ↑ BIM ↓	0.3	71
H3122	ALK-EML4		0.07-0.19	58-60,62,71-74
H3122-CR	Gatekeeper mutations*		0.24-0.26	58-60,62,71-74
H3122-CR	F1174L		0.62	58
H3122-CR1		EMT	>3	59,74
H3122-TR		EGFR ↑	2.56	58,71
DFCI076	ALK-EML4, L1152R		1.01	58
Ba/F3	ALK-ELM4		0.02-0.07	58, 60, 63, 64, 70
Ba/F3	Gatekeeper mutations**		0.2-0.9	58, 60, 63, 64, 70
SNU-2535	ALK-EML4, G1269A		8	61

wt: wild type
*: C1156Y, L1196M, G1269A, L1152R.
**: L1196M, G1202R, S1206Y, G1269S, 1151Tins, T1151K, L1152V, L1152R, C1156Y, I1171T, E1210K, F1245C.

3.2.2. EGFR

Activation of the EGFR signalling pathway, as another mechanism of resistance to ALK inhibitors, was demonstrated in sub clones of H3122 treated with crizotinib. Treatment of these H1322-CR cells with EGFR-TKI resulted in inhibition of cell growth[72,73]. Treatment of nude mice injected with H2228 NSCLC cells that overexpress the *EML4-ALK* fusion protein with crizotinib resulted in resistant xenografts that showed upregulation of the EGFR signalling pathway. These xenografts appeared to be sensitive to a combination of crizotinib and ganetespib. Ganetespib inhibits different HSP90 clients, including EGFR and may thus target the EGFR resistance associated activation in these cells and explain the enhanced sensitivity to the combined treatment. HSP90 inhibitors also directly have impact on ALK stability and therefore they are used to treat ALK resistant patients. Functional loss of the signalling cascades was also associated with increased BIM protein expression. Based on these findings the authors suggested that BIM and EGFR upregulation, without activating mutations, is associated with crizotinib resistance[59].

3.2.3. Autophagy

Another mechanism proposed in H3122-CR cells was the induction of autophagy. Inhibition of the autophagy pathway by chloroquine and bafilomycin showed increased sensitivity to crizotinib[74]. However, the clinical relevance of autophagy in the development of crizotinib resistance is not known.

3.2.4. Epithelial-mesenchymal transition (EMT)

Phenotypical alterations of the cell morphology due to down regulation of E-cadherin and *AXL*, and upregulation of vimentin are characteristics of EMT that have been implicated in crizotinib resistance. H2228 crizotinib resistant cells did not have secondary mutations, but did show epithelial-mesenchymal transition (EMT). EMT induced by TGF-β1 revealed resistance to crizotinib in lung cancer cell lines that was reversible by removal of the TGF-β. Suppression of vimentin in H2228-CR cells by siRNA treatment restored sensitivity to crizotinib[71]. Thus, EMT may be a mechanism of resistance to crizotinib treatment as well.

4. Concluding remarks

Afatinib, erlotinib and gefitinib are the main drugs used to target EGFR and crizotinib is the main drug to target ALK. Alternative TKIs are still under investigation in clinical studies. For EGFR, the T790M mutation is important in the development of resistance to reversible TKI. The current literature is contradictory about its role in irreversible EGFR blocking agents such as afatinib. Clinical relevant concentrations for afatinib (0.08μM) and dacomitinib (0.04μM) are much lower than those for both erlotinib (4μM) and gefitinib (0.9μM)[75-78]. In relation to the effective dose required to inhibit cell growth of T790M positive lung cancer cell lines, it is unlikely that clinical effects will be achieved with the EGFR-TKIs in patients with a secondary T790M mutation. Similar, there is also no support for a role of *HER2* amplification, although this has been observed as a resistance mechanism after first generation TKI. This is probably due to the fact that afatinib is a HER2 inhibitor as well.
One of the most evident afatinib resistance mechanisms is amplification of *MET* (Figure 3.1). Current studies indicate that resistance is not well associated with *MET* mutations or positive MET protein staining. The V843I mutation affecting binding of afatinib to EGFR causes resistance at least in cell lines. Overexpression of *FGFR1*, increased IL6R/JAK/STAT signalling, enhanced interference with the aerobic glycolysis, autophagy and SFK all have been associated with resistance to afatinib in one or more lung cancer cell lines. Until now, the only proven resistance mechanisms for afatinib include the V843I mutation and

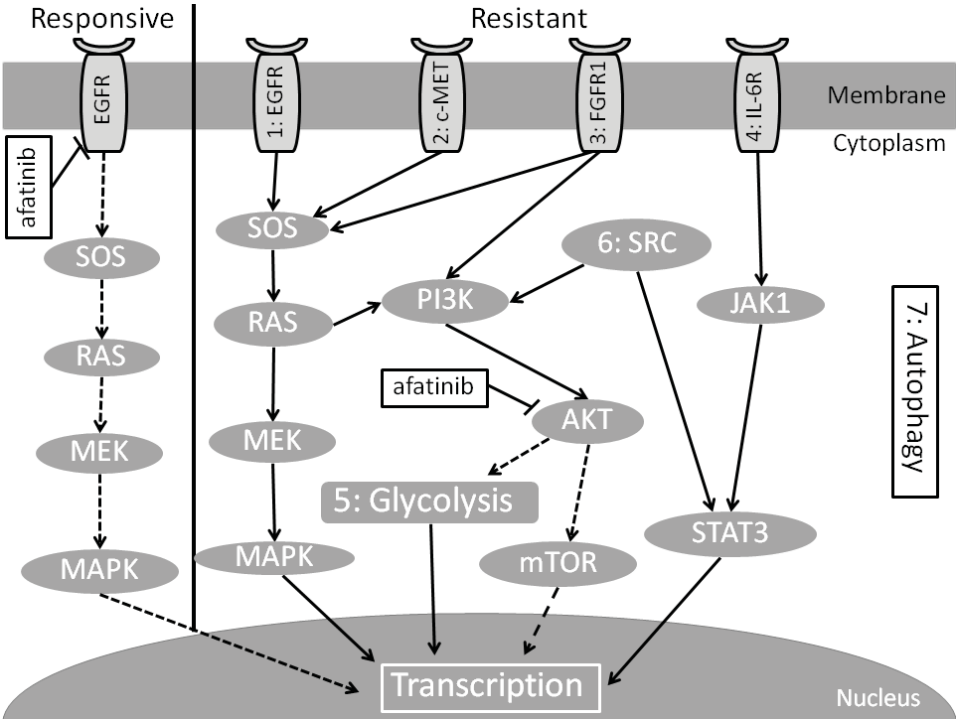

Figure 3.1. Overview of probable resistance mechanisms in EGFR-TKI afatinib.
Legend: Proven resistance mechanisms include gatekeeper mutations (1) and MET amplification (2). The V843I mutation is a proven mechanism of resistance while this is most likely not the case for the T790M mutation. *MET* amplification is a proven resistance mechanism, but *MET* mutations probably not. All other proposed mechanisms (3-7) need to be studied in more detail to proof their causal resistance in patient samples. *FGFR1* amplification (3), IL-6R/JAK1/STAT3 pathway (4) and autophagy (7) have been associated with resistance. The glycolysis pathway (5) induced by AKT has been proposed, although it is not clear why resistance occurs. Src upregulation (6) has been associated with resistance. Dashed arrow inhibited pathway, solid line arrow induced pathway.

MET amplification (Figure 3.1). Other mechanisms studied only in cell lines need to be confirmed in afatinib resistant tumour samples.

The main mechanism of crizotinib resistance is gain of a gatekeeper mutation leading to less effective binding of crizotinib to the mutated ALK kinase domain (Figure 3.2). This has been shown in both patient samples and in cell lines. As only 40% of patients with clinical resistance have such gatekeeper mutations, there have to be other mechanisms in the remaining 60% of patients. Analysis of re-biopsies of patients upon crizotinib resistance revealed *ALK* amplifications, *EGFR* and *KRAS* mutations.

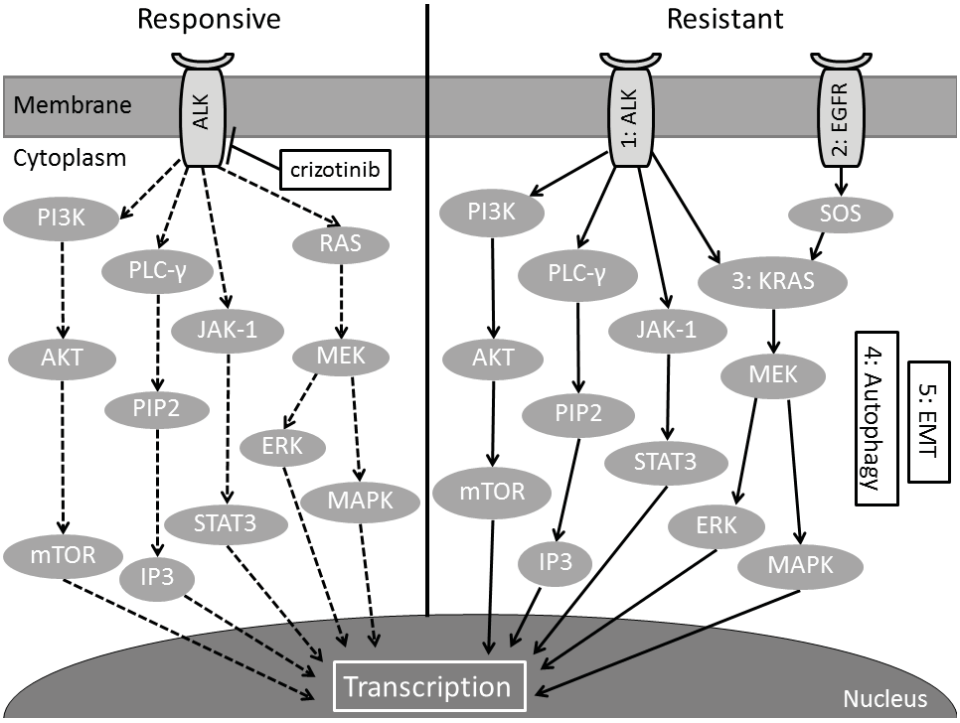

Figure 3.2. Overview of possible resistance mechanism induced upon treatment with the ALK inhibitor crizotinib.

Legend: The only proven resistance mechanisms is gain of ALK gatekeeper mutations (1) due to lower binding efficiency of crizotinib. Other possible resistance mechanisms include EGFR (2) upregulation which are associated with resistance to crizotinib in cell lines and mutations, which have been found in patients resistant to crizotinib; KRAS mutations (3) have been found in patients treated with afatinib; autophagy (4) has been found in a crizotinib resistant cell line; epithelial-mesenchymal transition (5) has been found in resistant cell lines to crizotinib. Possible clinical relevance of these mechanisms are not clear yet. Dashed arrow inhibited pathway, solid line arrow induced pathway.

The causal nature of these mutations needs to be confirmed and in a significant proportion of the patients the resistance mechanism remains unknown. Alternative mechanisms studied only in lung cancer cell lines include activation of the EGFR pathway, induction of the autophagy pathway and EMT. So, besides ALK gatekeeper mutations, all other proposed resistance mechanisms need to be studied in more detail to either proof the real cause of the observed resistance and whether these mechanisms also really determine resistance in patients treated with crizotinib *in vivo*.

4.1. Future perspectives

It is evident that in depth studies on the proposed resistance mechanisms are required to elucidate the full spectrum of TKI resistance. The discrepancy between clinical and cell line or xenograft results are due to the small therapeutic index of second generation EGFR TKI, such as afatinib. Clinical achievable doses of EGFR TKIs providing a balance between efficacy and side effects were too low to suppress T790M efficiently in EGFR mutant patients. Another known strategy to overcome resistance is to use a TKI drug holiday. During this drug holiday patients receive cytotoxic chemotherapy. After progression on this treatment, a re-challenge of TKIs can be given with the same TKI as used in first line, indicating that the initial resistance is not caused by mutations[79]. Prospective studies are currently being performed to define response rates using this strategy. Beside this, next generation TKIs have been developed to overcome resistance. Those smaller molecules fit better in the functional tyrosine kinase pocket than in the wild type pocket, e.g. osimertinib and rociletinib for EGFR mutation positive cases and ceritinib, alectinib, brigatinib and PF-06463922 for *ALK* translocations[2,3,69,80,81]. The renewed sensitivity towards TKIs after a drug holiday may support a role of epigenetic mechanisms, however, these mechanisms have not been studied yet for the TKIs discussed here in lung cancer. A study with the HDAC inhibitor entinostat showed that a subgroup of patients with EMT had an advantage in OS. Therefore, further biomarker studies are needed to define which patients will respond to adding HDAC inhibitors[82]. Resistance to EGFR-TKIs is associated with a *BIM* deletion polymorphism in cell lines. HDAC vorinostat restored the cell death pathway in cell lines with the *BIM* deletion and overcame resistance to gefitinib[83]. Moreover, HDAC inhibitors in combination with HSP90 inhibitors have a synergistic effect on inhibition of growth of TKI resistant cell lines[66]. This combination has not been tested in a clinical setting yet.

Next generation sequencing of resistant tumour samples and functional studies of observed recurrent alterations can help to explain the complex pattern of resistance after exposure towards TKI. Current studies suggest involvement of multiple pathways as possible resistance mechanisms and these should be functionally tested in cell lines to prove a causal relation. Novel gene editing techniques such as CRISPR-cas and TALENS may speed up elucidation of specific resistance mechanisms in cell lines that can be validated in patient derived xenografts for the different EGFR-TKIs and ALK inhibitors. Efficacy studies using combination therapy to prevent resistance to TKI may be a promising strategy for future clinical trials. Moreover, new therapeutic strategies are available to overcome the currently known resistance mechanisms.

5. References

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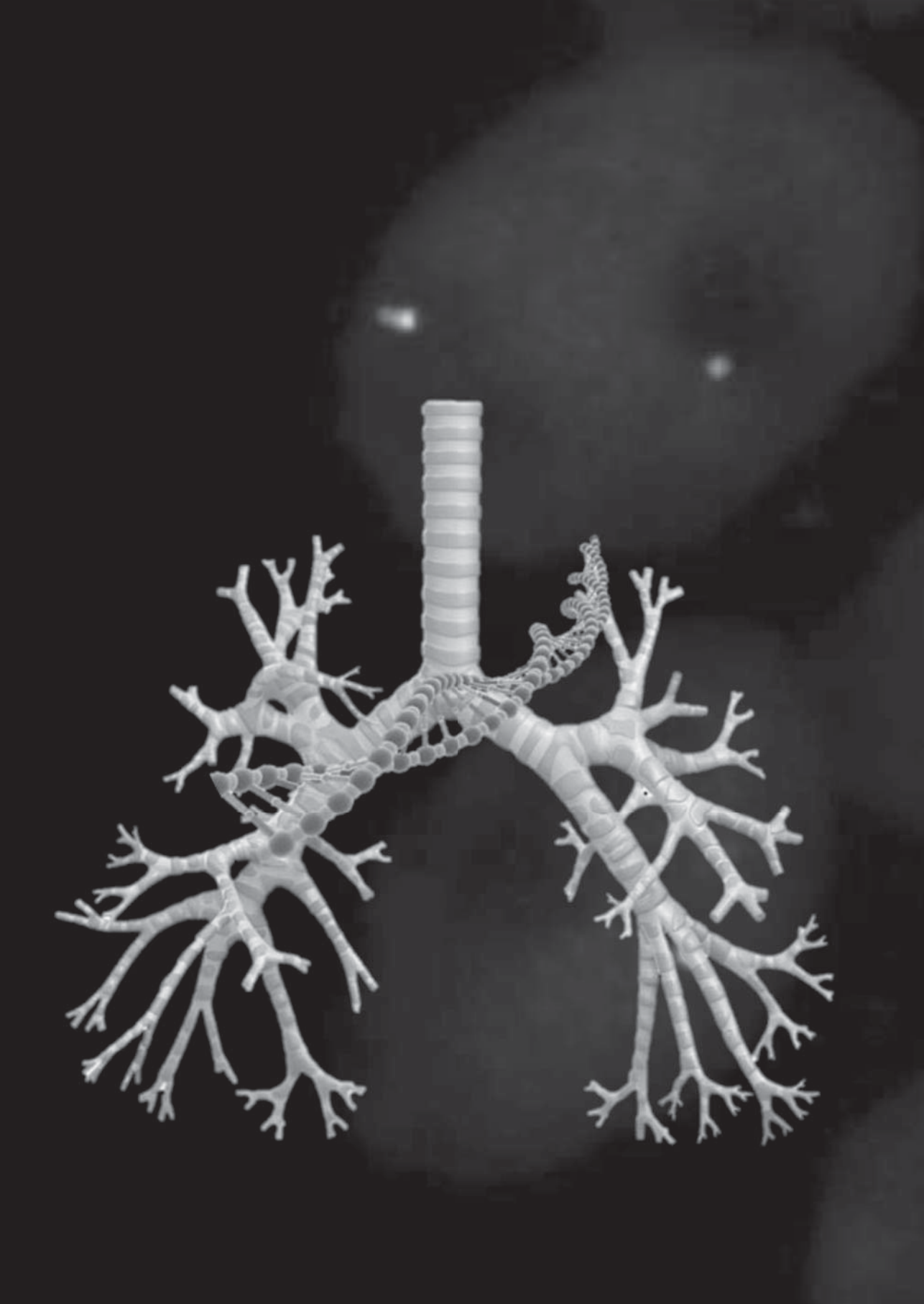

4

Overall survival in EGFR mutated non-small-cell lung cancer patients treated with afatinib after EGFR TKI and resistant mechanisms upon disease progression

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Submitted

Abstract

Purpose

To determine survival in afatinib-treated patients after treatment with first-generation EGFR tyrosine kinase inhibitors (TKIs) and to study resistance mechanisms in afatinib-resistant tumours.

Methods

Characteristics and survival of patients treated with afatinib after resistance to erlotinib or gefitinib in two large Dutch centres were collected. Whole exome sequencing (WES) and pathway analysis was performed on available pre- and post-afatinib tumour biopsies and normal tissue.

Results

A total of 38 patients were treated with afatinib. T790M mutations were identified in 22/29 (76%) pre-afatinib treatment tumour samples. No difference in median progression-free-survival (2.8 months (95% CI 2.3-3.3) and 2.7 months (95% CI 0.9-4.6), p=0.55) and median overall-survival (8.8 months (95% CI 4.2-13.4) and 3.6 months (95% CI 2.3-5.0), p=0.14) were observed in T790M+ patients compared to T790M- mutations.

Somatic mutations in *TP53*, *ADAMTS2*, *CNN2* and multiple genes in the Wnt and PI3K-AKT pathway were observed in post-afatinib tumours of six afatinib-responding and in one non-responding patient. No new *EGFR* mutations were found in the post-afatinib samples of the six responding patients. Further analyses of post-afatinib progressive tumours revealed 28 resistant specific mutations in six genes (*HLA-DRB1*, *AQP7*, *FAM198A*, *SEC31A*, *CNTLN*, and *ESX1*) in three afatinib responding patients. No known EGFR-TKI resistant-associated copy number gains were acquired in the post-afatinib samples.

Conclusion

No differences in survival were observed in patients with *EGFR*-T790M treated with afatinib compared to those without T790M. Tumours from patients who had progressive disease during afatinib treatment were enriched for mutations in genes involved in Wnt and PI3K-AKT pathways.

1. Introduction

Most patients with advanced non-small cell lung carcinoma (NSCLC) with epidermal growth factor receptor (EGFR) activating mutations will develop resistance after 6-9 months of treatment with first generation reversible tyrosine kinase inhibitors (TKIs) such as erlotinib or gefitinib[1,2]. The most common resistance mechanism is caused by the T790M gatekeeper mutation, and is detected in about half of the patients. Additional resistance-associated mechanisms are *MET* amplification, *HER2* mutations, transformation to small-cell lung cancer, expression of *IGFR1*, or alternative pathways to maintain PI3K/Akt signalling[3-7]. Because afatinib showed effectiveness in erlotinib resistant lung cancer models, afatinib effectiveness was studied in the Lux-Lung 4 study[8]. In this study patients with acquired resistance to first generation EGFR-TKIs exhibited a low response rate to afatinib and consequently the study did not meet its primary endpoint[9]. Reported resistance mechanisms to afatinib after first generation TKI are other mutations in *EGFR* (e.g. V843I), *FGFR1* amplification, upregulation of IL6R/JAK1/STAT3, glycolysis and Src pathways, and autophagy[10-17].

The T790M mutation plays a role as mechanism of resistance after first line treatment with afatinib[18]. However, in an Eastern Asian study, T790M played no role in treatment outcome or the prognosis of patients treated with second-line afatinib indicating a similar effect on both T790M positive and negative tumour clones[19]. The development of late occurring T790M clones in tumours may go along with other resistant mechanisms than early developing T790M clones.

In this study we analysed survival of mostly Caucasian patients treated with afatinib after becoming resistant to EGFR-TKI. In addition, we investigated the development of afatinib resistant associated mutations using whole exome sequencing (WES) in a subset of patients.

2. Materials and methods

2.1. Patient selection

Patients with relapsed advanced NSCLC whose tumour had progressed following initial disease control for more than 12 weeks with EGFR-TKI and subsequently treated with afatinib 40 mg daily, partly on a compassionate use program, were enrolled[20]. Patient characteristics including number of treatment lines, duration of previous EGFR-TKI exposure, the duration of afatinib use were recorded.

2.2. Tumour response

Tumour responses were assessed by comparing CT of chest and abdomen before start of afatinib, and every 6 weeks during treatment using RECIST version 1.1 criteria[21].

2.3. Tumour biopsies and diagnostic molecular analysis

Tumour biopsies were tested for the presence of *EGFR* mutations before and after treatment with EGFR-TKI. Re-biopsies were taken for WES prior to start of afatinib and upon subsequent tumour progression. Paired blood or normal tissue was used as control to filter for personal variants. Briefly, 3-micron paraffin embedded tumour tissue sections were stained with haematoxylin and eosin and assessed for tumour content. Subsequent tissue sections of 10 micron were used for DNA isolation. Diagnostic testing for mutations was performed using high resolution melting analysis (HRM) for *EGFR* exons 18, 19, 20 and 21(CCDS5514.1), for *KRAS* exon 2 for codon 12, 13, 61(CCDS8702.1) and for *BRAF* exon 15(NM_004333)[22,23]. PCR products with an abnormal HRM curve were re-amplified and subjected to Sanger sequencing to identify the mutation. *ALK* and *ROS1* translocations were determined by Abbott FISH tests (Abbott 06N38-020 and 08N29-020).

2.4. Whole exome sequencing

Samples with a tumour cell content of less than 50% were subjected to laser microdissection (LMD6000, Leica, Wetzlar, Germany). DNA from FFPE samples was isolated using ReliaPrep™ FFPE gDNAMiniprep System-kit (Promega, Madison, USA) following the protocol of the manufacturer. A standard salt-chloroform protocol was used to isolate DNA from blood cells. Quality control and WES were performed by BGI (BGI Tech Solutions Co. Ltd, Hong Kong). Raw image files were processed by Illumina base-calling Software 1.7 for base-calling with default parameters (Illumina Inc., San Diego, USA). For further details see supplementary file 1.

2.5. Identification of afatinib resistance associated mutations

Several criteria were used to identify afatinib resistance associated mutations, by e.g. excluding personal variants. We used different strategies to identify resistance-associated mutations. For details see supplementary file 1.

2.6. Pathway analysis

Partek Genomics Suite 6.6 (Partek Inc., St Louis, MO) was used to link mutated genes to either particular pathways only or whether they belonged to the same pathways.

2.7. WES-based copy number variant analysis (CNV)

Pseudo probe data were generated with VarScan2 and Samtools as described previously by Koboldt et al. and Li et al.[24,25]. CNV plots of the post-afatinib tumour were compared to the CNV plot of the pre-afatinib tumour of the same patient by a combination of calculated ratios and visual inspection.

2.8. Statistical analysis

Descriptive statistics were used for the patient characteristics. Objective tumour response rate (ORR) was defined as the best response to treatment of complete or partial response (CR/PR) according to RECIST 1.1[21]. PFS was defined as time from EGFR-TKI start until progressive disease (PD) according to RECIST 1.1 or death. OS was defined as the time from EGFR-TKI start until death or lost to follow up. Patients who had not progressed were censored at the last day of follow-up. PFS and OS were estimated with Kaplan-Meier survival curves using log-rank test for estimating group differences. Chi-square test was used to compare group variables. P-values <0.05 were considered significant. Statistical analyses were performed with SPSS-Statistics version 22.0 (IBM corporation, Armonk, NY, USA).

3. Results

3.1. Study population

Between April 2009 and January 2014, 38 patients from two Dutch university hospitals were treated with afatinib (Supplementary Figure 4.1). Follow-up was more than 18 months after the last patient was included. All patients received gefitinib or erlotinib prior to afatinib, two patients received erlotinib, followed by gefitinib. A platinum doublet was given as first line treatment to 24 patients before treatment with first generation TKI and afatinib (Figure 4.1).

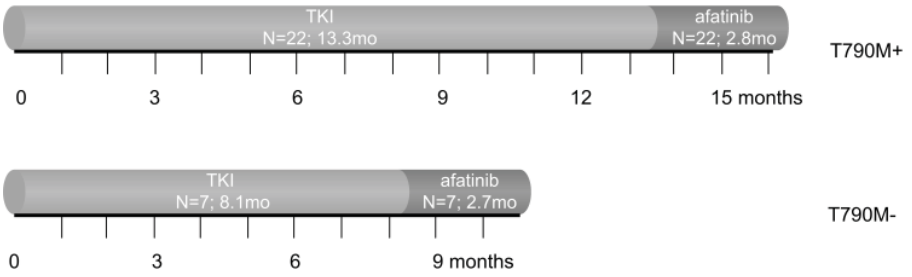

Figure 4.1. Median PFS for sequential treatments in T790M positive and negative NSCLC patients.
After first generation EGFR-TKI, 22 patients had a T790M and 7 did not. All patients received afatinib, afterwards.

3.2. Efficacy of 1st line and 2nd line TKI treatments

Median PFS on first-line erlotinib or gefitinib TKI treatment in those who turned out to be T790M positive (n=22) and negative (n=7) in later biopsies showed a trend to be different, 13.3 months (95% CI, 10-17) and 8.1 months (95% CI, 0-16) respectively (p=0.06; Figure 4.1). Tumour response rate of all 38 patients on second line afatinib was 18% and the disease control rate was 79%. Median PFS on afatinib was 2.8 months (95% CI, 2.3-3.2) and median OS was 6.9 months (95% CI, 1.5-12.4).

3.3. Survival by mutation type in afatinib treated patients

Median PFS of afatinib treated patients with (n=22) and without (n=7) T790M mutation was similar with 2.8 months (95% CI 2.3-3.3) and 2.7 months (95% CI, 0.9-4.6), respectively (p=0.55; Figure 4.1). Median OS was numerical better in the T790M positive as compared to the T790M negative group, although not significant (8.8 months (95% CI, 4.2-13.4) and 3.6 months (95% CI, 2.3-5.0); p=0.14; Figure 4.2).

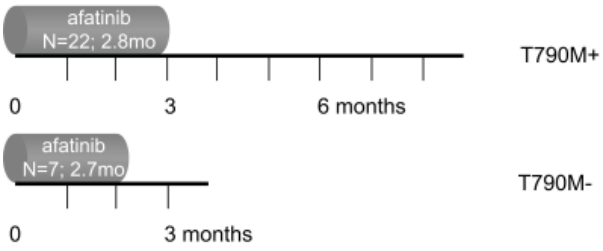

Figure 4.2. Median PFS and OS for sequential treatments in T790M positive and negative advanced NSCLC patients.
Survival outcome of afatinib treatment is shown after first generation EGFR-TKI treatment. The line represents the overall survival. The bars indicate the progression free survival for afatinib.

3.4. EGFR mutational analysis

The first biopsy was taken before start of any TKI treatment (n=38). Thirty-three patients had activating *EGFR* mutations, the most common mutation was a deletion in exon 19 (87%; n=24; Table 4.1); no T790M mutations were observed in any of the biopsies taken before EGFR-TKI. Four patients with wild type *EGFR* and one patient for which mutation analysis was not possible were included based on Jackman's criteria (TKI treatment with at least stable disease for 6 months)[20]. None of these five patients had *KRAS* and *BRAF* mutations or *ALK* and *ROS1* translocations.

Table 4.1. EGFR mutation status in tumour biopsies of a cohort of 38 advanced NSCLC patients

EGFR mutation	First biopsy (n=38)	Pre-afatinib biopsy (n=33)	Post-afatinib biopsy (N=18)	WES (N=7)
Exon 18	1	0	1	0
Exon 19	24	4	5	2
Exon 19 + T790M		18	6	1
WT				1
Exon 21	7	2	2	0
Exon 21 + T790M		4	2	2
Exon 18 + 20	1	1	0	0
WT	4	4	2	0
Exon 18 + 21 +T790M				1
Mutation analysis not possible	1	0	0	0

A diagnostic biopsy taken before erlotinib and/or gefitinib treatment is called first biopsy; A biopsy after first generation EGFR-TKI is called pre-afatinib biopsy. Biopsy taken in patients who responded to afatinib and underwent a biopsy afterwards is called post-afatinib biopsy. WT: wild type.

A second biopsy taken after failure on erlotinib or gefitinib was available of 29 *EGFR*+ patients. In 22/29 (76%) patients with a known activating *EGFR* mutation the T790M mutation was detected as a second mutation. The presence of a T790M mutation was significantly more common in patients treated with erlotinib alone (18/19; 95%) compared to patients treated with gefitinib alone (2/8; 25%, $p<0.001$; Table 4.2). The two patients treated with erlotinib and gefitinib were excluded from this comparison.

Table 4.2. Patient characteristics of the afatinib treated group according to T790M mutation

	T790M + (N=22)	T790M - (N=7)	P-value
Median age (years; range)	60 (32-81)	56 (44-67)	0.45
Sex			0.55
Male	4	2	
Female	18	5	
ECOG Performance status			0.12
0	12	1	
1	9	6	
2	1	0	
Ethnic origin			0.49
Caucasian	19	7	
Asian	2	0	
Afro-American	1	0	
Smoking history (at start afatinib treatment)			0.33
Never smoker	10	5	
Ex-smoker	10	1	
Current smoker	0	0	
Unknown	2	1	
Number of lines of previous chemotherapy			0.29
0	11	1	
1	3	4	
2	6	2	
>2	2	0	
EGFR TKI before afatinib			<0.001
Erlotinib	18	1	
Gefitinib	2	6	
Both subsequently	4	1	

3.5. Whole exome sequencing (WES)

Out of the 18 patients with a post-afatinib biopsy, there was enough tumour tissue to perform WES in six patients. Normal, pre- and post-afatinib samples were available in 3 of the 6 responding patients (#2, 5 and 6) and only normal and post-afatinib samples with sufficient DNA quality were available from the other three patients (#1, 4 and 7). In only one (patient #3) out of nine non-responders to afatinib, there was enough tumour tissue in the pre-afatinib biopsy. Of the non-responding patient we analysed pre-afatinib normal and tumour tissue samples.

In the initial analyses we focused on recurrently mutated genes found in at least 2 of the 7 biopsies irrespective of presence of the mutation in the pre-treatment biopsy. This revealed presence of 284 mutations in 68 genes (Supplementary Table 4.1).

According to putative damaging effect according to CADD, a high score (>20) was found for 27 mutations in 25 genes, e.g. *TP53*, *DYNC2H1*, *MGA*, *USH2A*, *ROBO2*, *TEX15*, *ADAMTS2*, *CUL7*, *IL33*, *ADAMTS12*, *CNN2*, *CNKSR3*, *LAMA3*, *EML6*, *TTN*, *KNDC1*, *EPB41*, *PKHD1L1*, *KLHL23*, *EVPL*, *CACNA2D1*, *CDC27*, *KCNT2*, *ASTN2* and *MROH2B*. A moderate CADD score (10-20) and/or recurrent mutations were found for 110 mutations in 45 genes, e.g., *OR8U1*, *MUC16*, *MUC6*, *ANKRD36C*, *HLA-DRB5*, *DYNC2H1*, *NEFH*, *FASLG*, *MUC5B*, *PRSS3*, *HYDIN*, *MGA*, *USH2A*, *TAS2R43*, *GRP98*, *C4BPB*, *DOCK2*, *INVS*, *CUL7*, *NHSL2*, *USP24*, *TPSB2*, *MUC12*, *OR2T4*, *CNN2*, *CNKSR3*, *LAMA3*, *TTN*, *KNDC1*, *EPB41*, *EVPL*, *FZR1*, *CACNA2D1*, *CDC27*, *KCNT2*, *EMP2*, *MST1*, *ARHGAP32*, *NLGN4X*, *HLADQA1*, *HERC2*, *ASTN2*, *SP8*, *PRSS1* and *MROH2B*. *CNN2* is the only gene with an identical high CADD score mutation in 2 patients. Some of the other recurrently mutated genes had mixed high and moderate CADD scores. In total, 137 mutations in 48 genes were identified as potentially involved in afatinib resistance.

In patient #7 treated with afatinib according to Jackman's criteria, without a known EGFR mutation, WES revealed activation mutations in *EGFR*, e.g. G719C and a L861Q. In the other six patients, no new *EGFR* mutations were identified. For the three pre-afatinib samples this was consistent with the targeted diagnostic mutation tests.

In patients #2, 5, and 6 (Table 4.3) WES data of normal tissue, pre- and post-afatinib tumour samples could be analysed. Four hundred forty five mutations in 367 genes (range 87–216) had higher MRF, or were specific, for the post-afatinib treatment sample.

Mutations in these genes might have contributed to the observed afatinib resistance. Six genes, with in total 28 mutations, were recurrently mutated in at least two out of three patients, i.e. *HLA-DRB1*, *AQP7*, *FAM198A*, *SEC31A*, *CNTLN*, and *ESX1*.

Most of the mutations observed in *HLA-DRB1* were also present in the ExAc database and therefore appear to be less important. The other mutations, absent in the ExAc database but present in the COSMIC database (in different tumour types), might be relevant, such as p.W38fs* in *HLA-DRB1*, p.Q30fs* in *AQP7*, p.C135R in *TP53*, p.Q220* in *HLA-DRB5*, p.G8V in *PRSS3*, p.S1155R in *USH2A* and p.V521I in *KCNT2*. The p.K41T mutation in *HLA-DRB1*, p.IT255T and p.Q136E in *TP53* and p.P2811S in *USH2A* are also described in human lung cancer samples. The p.P95S mutation in *AQP7* was observed in patients #1 and #5.

Pathway analysis of all genes mutated in any of the 7 analysed patients indicated that most of the mutated genes were members of Wnt signalling (Supplementary Figure 4.2)

Table 4.3. Recurrently mutated genes in tumours from patients who progressed under afatinib

Gene	Patient	Chrom.	Pos.	REF	ALT	AA change	CADD score
HLA-DRB1	#2	6	32552130	C	A	R42S	0.145
			32552131	C	G	R42T	0.005
	#6		32552134	T	G	K41T	10.93
	#3		32552130	C	A	R42S	0.145
			32552132	T	A	R42W	10.52
	#4		32548544	T	G	I248L	16.04
	#7		32552130	C	A	R42S	0.145
			32552131	C	G	R42T	0.005
			32552137	G	C	P40R	0
			32552138	G	C	P40A	0.005
			32552143	C	T	W38*	13.84
			32552144	A	C	W38G	3.518
AQP7	#2	9	33385808	G	T	N194K	15.08
	#5		33385709	C	T	V96I	9.787
			33385712	G	A	P95S	10.22
			33385690	C	T	R234S	14.5
			33385698	A	G	L231P	16.61
	#1		33385712	G	A	P95S	10.22
	#4		33395131	TG	T	Q30	NA
FAM198A	#2	3	43074734	C	A	P327T	9.704
	#6		43074337	G	T	W194C	14.09
SEC31A	#5	4	83803067	C	T	R8H	27.8
	#6		83784534	CT	C	E482	NA
CNTLN	#2	9	17366624	TGAA	T	E633	NA
	#5		17236574	TG	T	A280	NA
			17236576	C	A	A280E	5.077
ESX1	#2	X	103497493	G	C	R175G	11.68
	#6		103498982	C	A	G120V	14.79

Chrom, chromosome; Pos, basepair location; REF/ALT, reference and altered nucleotides at mutated position; AA change, amino acid change; CADD, Combined Annotation Dependent Depletion score.

and PI3K-AKT (Supplementary Figure 4.3) pathways. In addition, we observed mutations in two genes of the glycolysis pathway (Table 4.4). We did not identify mutations in genes related to the pathways known to be associated with afatinib resistance, e.g. autophagy and IL6R/JAK/STAT.

Table 4.4. Overview of significantly involved pathways in patients' progressive disease on afatinib and the involved mutated genes

Patient sample	N/P/R						N/R			N/P
	#2		#5		#6		#1	#4	#7	#3
	A	B	A	B	A	B	C	C	C	D
Pathway										
Glycolysis					ADPGK			LDH6A		
Autophagy										
IL6/JAK/STAT										
Wnt	MAPK8	TP53 LEF1		TP53	FOSL1	PLCB4	WNT9B *TP53* FZD10 APC2	INVS	AXIN1 *TP53*	*TP53*
PI3K-AKT	COL4A4	ITGA2 EGFR VWF TSC2 TP53	PPP2R2B	EGFR PIK3CA PIK3CG COL4A5	LAMC3	EGF EGFR COL1A2	*EGFR* LAMB3 PTEN FASLG *TP53*	*EGFR* FASLG	*TP53*	*KDR* RELN FASLG *TP53*

N: normal tissue or buffy coat, P: biopsy before afatinib treatment, R: biopsy after afatinib treatment. A: resistant biopsy specific based on MRF R >2x MRF P, B: mutation present in both pre and post afatinib biopsy based on MRF R <2x MRF P and MRF R > 0. C: mutation in resistant biopsy based on MRF R > 0, D: mutation in pre-afatinib biopsy in non-responding patient based on MRF P > 0; *italic*: present in other primary samples.

3.6. Copy Number Variations

We observed only a few differences in copy number variations (CNV) between the pre- and post-afatinib samples. In patient #2 copy number gain (CNG) of part of chromosome 5, 8, 11 and 16 and loss of part of chromosome 4 and 14 was observed (Supplementary Figure 4.4A). In patient #5 no differences in CNV between pre- and post-afatinib biopsies was observed (Supplementary Figure 4.4B). In patient #6, copy number loss was seen only for part of chromosome X (Supplementary Figure 4.4C). Specifically, no CNV aberrations in *MET*, *FGFR1*, Src or genes involved in the IL6/JAK1/STAT3 pathway were found.

4. Discussion

In this study we investigated afatinib resistance in patients with relapsed advanced NSCLC whose tumour had progressed on EGFR-TKI and subsequently were treated with afatinib. In 38 patients with an *EGFR* mutation or treated with TKIs according to Jackman's criteria we first determined the prevalence of T790M mutations in *EGFR* upon treatment with either erlotinib or gefitinib. The exon 20 T790M mutation was detected under EGFR-TKI selection pressure in re-biopsies of 22/29 (76%) patients in our cohort, which is slightly higher than reported in literature (25-63%)[26-28]. The percentage of T790M+ patients was significantly higher in the erlotinib treated as compared to the gefitinib treated patients. In the literature there is a trend that T790M mutations are numerically higher in patients who received erlotinib[29]. The duration of first line reversible TKIs did not influence the occurrence of a T790M mutation. In our cohort of afatinib treated patients, PFS (2.8 months) and OS (9.2 months) were similar to the Lux-Lung 1 study[30]. We did not find an influence of the occurrence of T790M mutation on response outcome (PFS or OS) on afatinib treatment. To understand molecular events underlying progression of disease on afatinib treatment, WES was performed in all patients with sufficient tumour tissue to identify known and novel resistance mechanisms. We observed 68 recurrently mutated genes in 7 different patients with progression under afatinib, of which 137 mutations in 48 genes might be involved in afatinib resistance based on moderate or high CADD score. The R287Q mutation in *CNN2* is noteworthy based on the high CADD score and being identified in two patients. *CNN2* has been described in prostate and rectal cancer and is involved in cell migration and cell morphology[31,32]. Post-afatinib specific mutations were observed in *HLA-DRB1*, *AQP7*, *FAM198A*, *SEC31A*, *CNTLN* and *ESX1*.

The observed resistance associated mutations were present in a broad range of genes. Therefore, we explored if these genes clustered in specific pathways that might play a

role in afatinib treatment progression. We found that a substantial proportion of the mutated genes were part of the Wnt and/or PI3K-AKT pathways. Wnt pathway mutated genes were also implicated in erlotinib resistance in *EGFR* mutation positive lung cancer cell lines[33,34]. Inhibiting the Wnt pathway is attractive in treatment of resistant cancers and is now tested in phase I trials in cancer. The PI3K-AKT pathway has not been associated with afatinib resistance previously, except perhaps the hint observed in gefitinib resistant NSCLC patients treated with Paris Saponins, which induced apoptosis via the PI3K-AKT pathway in the tumour cells[35].

CNVs of different genes have been associated with resistance in *EGFR* mutant lung cancer[36]. However, in our cohort of afatinib resistant patients, no known afatinib resistance-related CNVs were observed. Together with the WES data, this suggests that in our patients, mutations in *EGFR*, *IGFR1*, *SRC* or in the IL6R/JAK1/STAT3 pathway, or genomic aberrations in *MET* or *FGFR1*, previously reported as resistance mechanisms by association in few patients or in cell lines, were not observed in our study to be involved in afatinib resistance[5,10].

In conclusion, no differences in survival were observed in patients with EGFR T790M treated with afatinib compared to those without T790M. Potential mechanism of resistance to afatinib treatment might be related to mutations in *HLA-DRB1*, *AQP7*, *TP53*, *HLA-DRB5*, *PRSS3*, *USH2A*, *KCNT2* and *CNN2* and to mutations in genes of the Wnt and PI3K-AKT pathways.

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6. Supplementary data

6.1. Whole exome sequencing (in more detail)

Reads were aligned to the human 1000 genomes reference based on the GRCh37 build using BWA 5.9rc[1]. Picard tools were used for format conversion and marking duplicate reads. Genome Analysis Toolkit (GATK) was used for indel realignment and base score quality recalibration (BSQR) by Molgenis Compute 4[2,3]. After using custom scripts in the VCF tools library, variant calling was performed using the GATK unified genotype and variant annotation by using SNPEFF/SNPSIFT 3.5 with the ensembl release 74 gene annotations (http://www.ensembl.org/index.html), dbNSFP2.3, and GATK with annotations from the Database of Single Nucleotide Polymorphisms (dbSNP) Bethesda (MD): National Centre for Biotechnology Information, National Library of Medicine (dbSNP Build ID: 137) and CosmicCodingMuts_v62[4-7]. For mutations with a moderate impact according to SNPEFF, we used the CADD value to discriminate between mutations with a possible (CADD score >10) or a probable effect (CADD >20) on protein function.

6.2. Identification of afatinib resistance associated mutations (in more detail)

Different criteria were used to identify mutations associated with resistance to afatinib treatment. First, we eliminated variants with a total read count of less than 10 in corresponding normal DNA, as we were not able to exclude them as personal variants (step 1). Then, we excluded germ line variants based on mutant read count of more than one and a total read count of 10-49, or mutant read count of more than two and a total read count of ≥50 in the normal DNA (steps 2 and 3). The remaining variants were regarded as true somatic mutations. Next, we filtered out variants with less than 10x coverage in either primary or resistant biopsies (step 4), as read counts for these variants are too low to be used for identification of afatinib resistance associated mutations. As we did not have pre-afatinib tumour sample for all seven patients, that also had post-afatinib samples, we followed two different strategies to identify potential resistance-related mutations: a) for all seven patients with adequate tumour samples we generated a list of genes having a mutation in the resistant sample irrespective of having a pre-afatinib sample or not, b) for 3 out of 7 patients with both pre- and post-afatinib samples, we selected variants with a more than two times higher mutant read frequency (MRF) in the resistant versus the primary biopsy ($MRF_R > 2*MRF_P$; step 5). In the final step of both analyses, we only included variants with a mapping quality >20 and

a quality score >20. Genes found in this analysis were browsed in the Exome Aggregation Consortium (ExAC), Cambridge, MA (URL: http://exac.broadinstitute.org) [accessed JUL-2016] to screen for any remaining known single nucleotide variants (SNVs). The COSMIC database was used to compare identified mutations in our cohort to the reported somatic mutations in cancer (http://cancer.sanger.ac.uk/cosmic) [accessed AUG-2016].

6.3. WES-based copy number variant analysis (in more detail)

Pseudo probe data were generated with VarScan2 and Samtools as described previously by Koboldt et al. and Li et al.[8,9]. Briefly, for each sample the pseudo probe derived GC-normalized log2 copy number ratios were generated by dividing the read counts of the tumour sample by the read counts of the corresponding normal sample. All alignments with a mapping quality greater than 40 in combination with a minimal segment size of 2kb and a maximal segment size of 5kb with a mean coverage of at least one were used to calculate the log2 ratios. CNV plots of the post-afatinib tumour were compared to the CNV plot of the pre-afatinib tumour of the same patient by a combination of calculated ratios and visual inspection.

6.4. References

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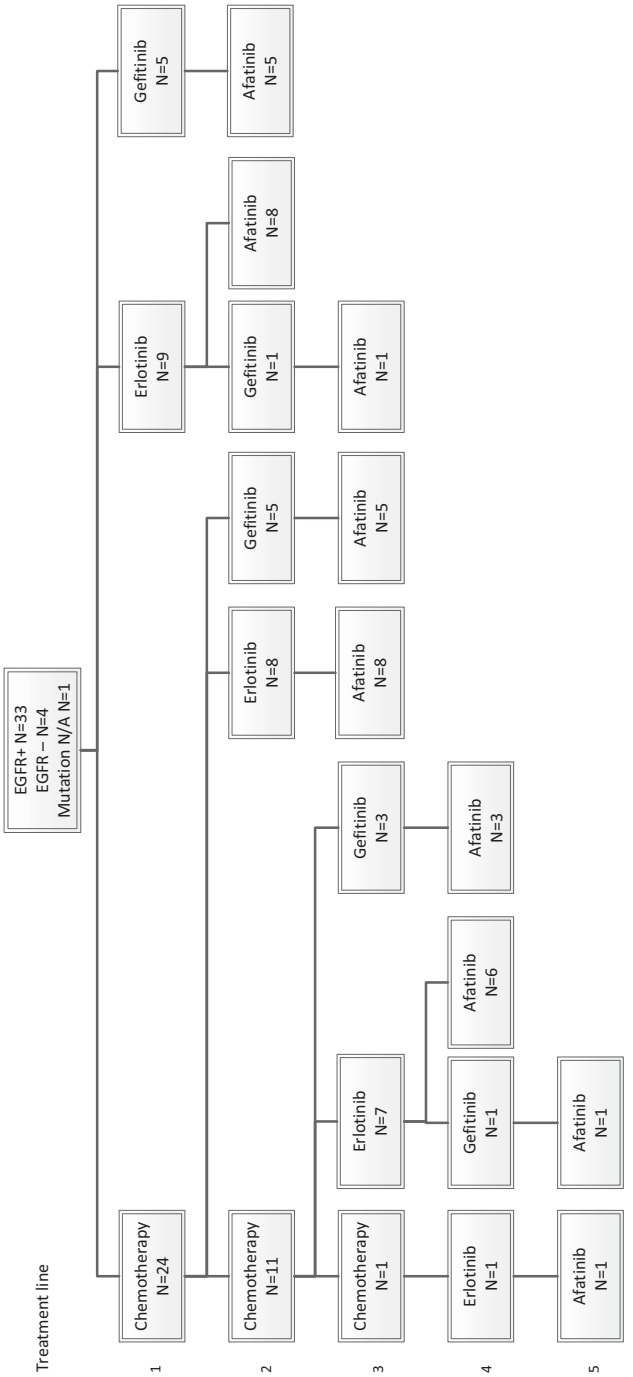
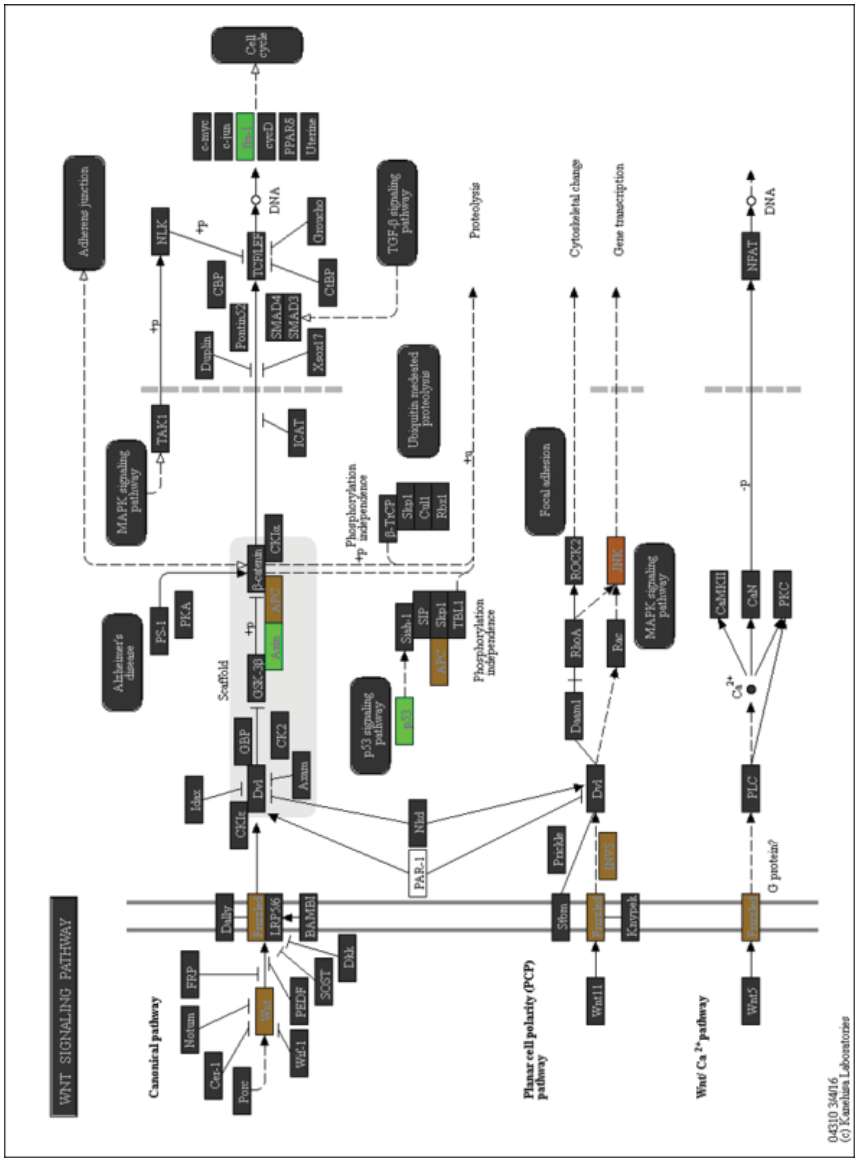
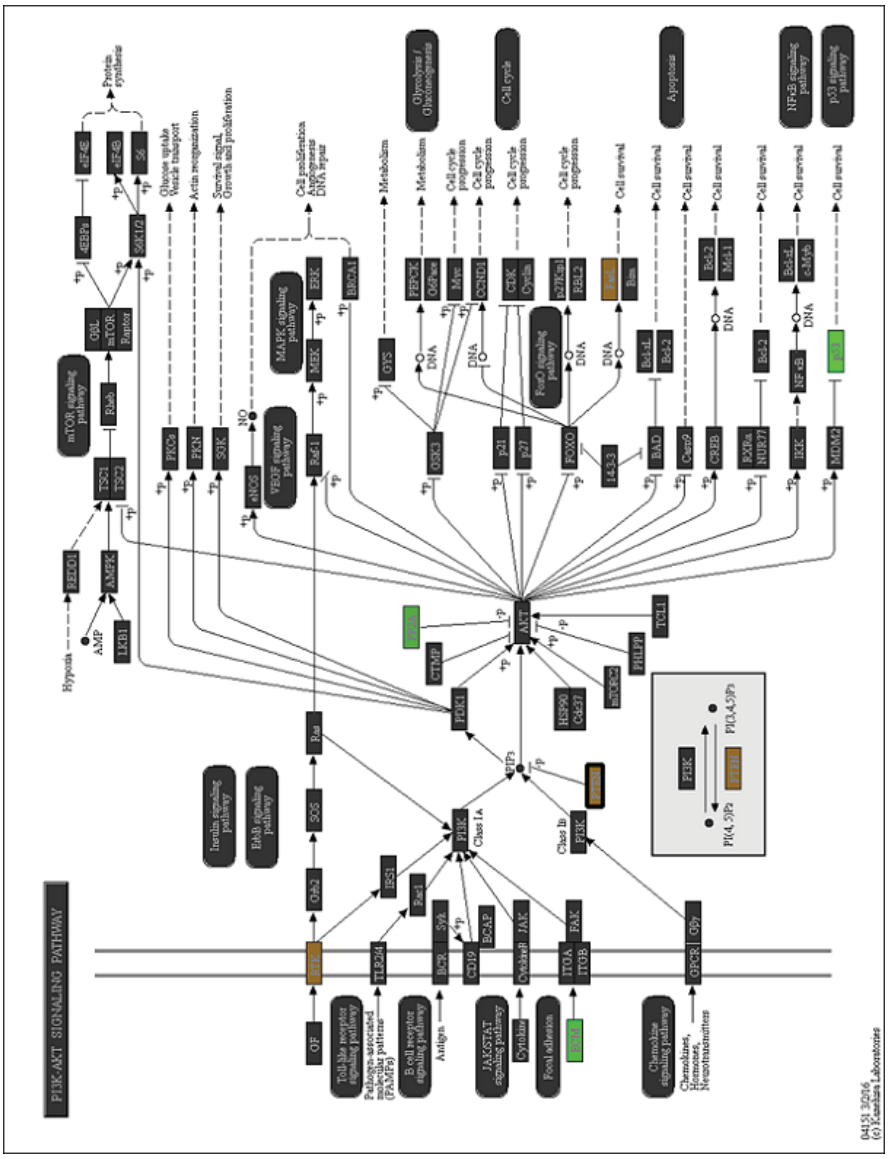
7. Supplementary figures



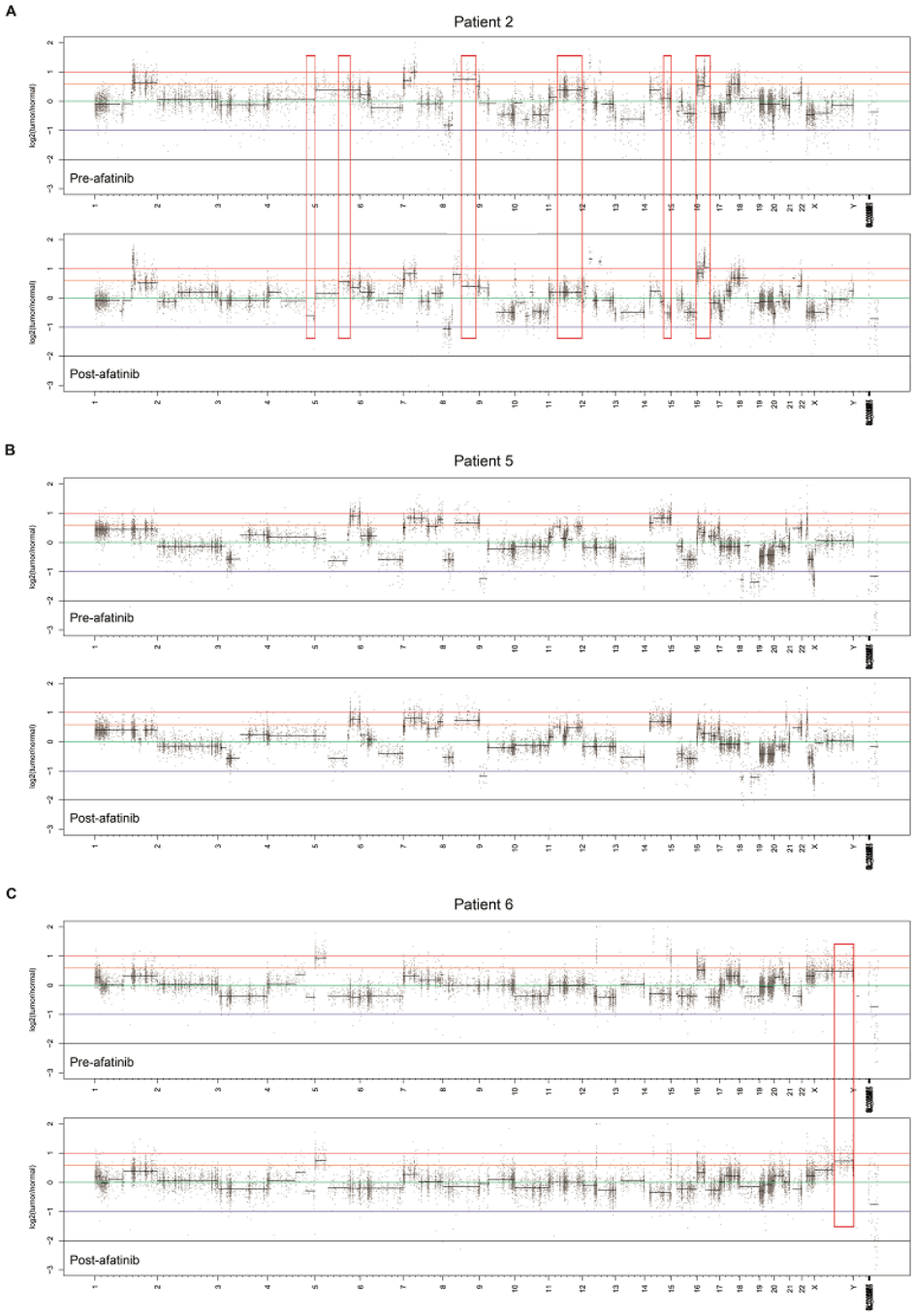
Supplementary Figure 4.1. Overview of treatment in 38 patients administered per treatment line.
Organogram of 38 treated patients where afatinib is given in different treatment lines (1-5). Chemotherapy was variable, e.g. cisplatinum/pemetrexed, carboplatinum/paclitaxel/bevacizumab, docetaxel, pemetrexed.



Supplementary Figure 4.2. Wnt signalling pathway involved in afatinib resistant tumours. Colour boxes (green, red, brown) are the different mutations found in different samples.
Multiple Wnt pathway associated genes are mutated.



Supplementary Figure 4.3. PIK3-AKT signalling pathway involved in afatinib resistant tumours.
Colour boxes (green, brown) indicate the genes that have been found to be mutated in at least one of the samples.



Supplementary Figure 4.4. Overview of copy numbers variations in patients #2, #5 and #6.
For each patients a CNV plot was generated of the pre-afatinib (top) and post-afatinib biopsies (bottom) in patients 2 (A), 5 (B) and 6 (C). The boxes indicate aberrations between pre-afatinib and post-afatinib biopsies with either copy number gain or loss

8. Supplementary table

Supplementary Table 4.1. Recurrently mutated genes in tumor samples from patients resistant to afatinib without information from primary biopsies using whole exome sequencing

GENE	PATIENT	CHROM	POS	REF	ALT	AA CHANGE	CADD score
OR8U1	#1	11	56143987	G	T	E296D	0.277
	#2		56143394	A	G	T99A	0.45
	#3		56143556	T	A	F153I	8.131
			56143562	A	G	M155V	0.014
			56143370	G	A	V91I	0.303
	#4		56143250	G	A	A51T	1.371
			56143257	C	A	T53K	4.977
	#7		56143103	G	A	A2T	12.13
			56143108	C	G	H3Q	0.249
EGFR	#1	7	55242464	AGGAATTAAGAGAAGC	A	KELREA745K	NA
	#2		55249071	C	T	T790M	30
	#3		55242464	AGGAATTAAGAGAAGC	A	KELREA745K	NA
	#4		55242465	GGAATTAAGA	G	ELR746	NA
			55242478	G	C	A750P	21.8
			55249071	C	T	T790M	30
	#5		55249071	C	T	T790M	30
			55259515	T	G	L858R	25.1
	#6		55249071	C	T	T790M	30
			55259515	T	G	L858R	25.1

TP53	#1	17	7577514	GTGA	G	IT255T	NA
	#3		7578527	A	G	C135R	22.8
	#7		7578524	G	C	Q136E	26.1
MUC16	#1	19	9064752	T	A	K7565I	4.321
	#3		9002623	C	T	R13398H	8.006
			9006764	T	G	T13162P	2.395
	#4		9002612	T	G	K13402Q	6.712
	#7		9008344	A	G	S13070P	1.811
			9027548	G	A	T12199I	10.15
			9027560	C	T	S12195N	6.373
MUC6	#2	11	1018408	G	T	P1465T	3.885
	#3		1018393	T	C	M1470V	1.88
	#4		1018218	T	G	H1528P	4.587
	#6		1016055	G	T	P2249Q	12.76
			1018218	T	G	H1528P	4.587
	#7		1018088	TG	T	P1571	NA
			1018093	G	GT	P1569P?	NA

Supplementary Table 4.1 continues on next page

Supplementary Table 4.1. ***Continued***

GENE	PATIENT	CHROM	POS	REF	ALT	AA CHANGE	CADD score
ANKRD36C	#3	2	96525702	C	T	S1268N	0.387
	#4		96610410	T	A	N486Y	5.517
			96610413	C	T	E485K	9.365
			96610420	G	T	D482E	4.959
	#5		96619713	C	A	E393*	12.16
	#7		96616501	C	CAA	V430V?	NA
			96616505	AGC	A	A429	NA
HLA-DRB5	#4	6	32487215	C	CT	R195Q?	NA
			32489855	T	A	D66V	9.422
			32489874	T	A	I60F	0.049
			32489876	T	A	D59V	11.35
	#5		32487153	C	G	E216Q	7.567
			32487158	G	GCA	T214M?	NA
			32487215	C	CT	R195Q?	NA
			32489882	T	A	H57L	12.16
			32489888	A	T	F55Y	5.704
			32489889	A	T	F55I	14.14
			32489892	G	A	R54W	8.85
			32489913	A	G	F47L	14
	#6		32487314	C	T	R162Q	12.82

HLA-DRB5	#7		32486438	G	A	Q220*	18.08
			32487353	T	C	N149S	0.054
			32489855	T	A	D66V	9.422
			32489882	T	A	H57L	12.16
			32489883	G	C	H57D	0.023
			32489888	A	T	F55Y	5.704
			32489889	A	T	F55I	14.14
DYNC2H1	#1	11	103052596	T	A	L2153*	47
	#2		102995846	G	A	R560Q	13.6
			103027318	C	CTGATTGTAA	P1316LIVT	NA
	#6		103126213	GAAGAAGAT	G	EED3433	NA
NEFH	#1	22	29885638	T	A	V670E	0
	#2		29885644	C	A	A672E	0.001
	#7		29885644	C	A	A672E	0.001

Supplementary Table 4.1 continues on next page

Supplementary Table 4.1. *Continued*

GENE	PATIENT	CHROM	POS	REF	ALT	AA CHANGE	CADD score
FASLG	#1	1	172635091	G	A	V261I	14.83
			172635097	G	C	E263Q	8.329
			172635109	G	A	V267I	12.67
			172635127	C	A	Q273K	0.888
	#3		172634812	ATTGT	A	IV168	NA
			172634817	C	CCGCT	L170RS?	0.334
			172634821	C	A	L171I	0.334
			172635109	G	A	V267I	12.67
			172635127	C	A	Q273K	0.888
	#4		172634812	ATTGT	A	IV168	NA
			172634817	C	CCGCT	L170RS?	NA
			172634821	C	A	L171I	0.334
MUC5B	#1	11	1260248	G	A	D1152N	5.604
			1260249	A	G	D1152G	3.325
			1260252	C	T	T1153I	8.179
	#3		1260248	G	A	D1152N	5.604
			1260249	A	G	D1152G	3.325

PRSS3	#1	9	33794797	TGA	T	MR3	NA
	#4		33794797	TGA	T	MR3	NA
			33794809	G	A	S7N	0.008
			33794812	G	T	G8V	0.033
			33796644	C	G	A72G	14.93
			33796649	C	T	P74S	11.42
			33796686	C	A	T86N	8.398
			33796703	C	G	L92V	0.246
			33796746	G	T	G106V	13.8
			33796758	T	A	I110N	13.82
			33796762	C	G	S111R	5.004
			33796766	C	T	Q113*	7.996
	#5		33794797	TGA	T	MR3	NA
			33794809	G	A	S7N	0.008
			33794812	G	T	G8V	0.033
HYDIN	#1	16	70841941	CTGCG	C	HA4968	NA
	#4		70902609	C	T	R3725Q	7.379
	#6		70852376	G	T	P4843T	12.98
MGA	#1	15	42054444	G	A	R2543K	18.68
	#4		41999929	G	T	G731V	25.7
	#6		41961989	G	GT	S300F?	NA

Supplementary Table 4.1 continues on next page

Supplementary Table 4.1. ***Continued***

GENE	PATIENT	CHROM	POS	REF	ALT	AA CHANGE	CADD score
HNRNPCL1	#3	1	12907518	T	A	S209C	8.642
			12907519	C	A	Q208H	7.204
	#4		12907284	T	C	T287A	0.001
			12907285	G	C	S286R	0.23
	#7		12907446	C	T	E233K	6.762
			12907457	A	G	M229T	0
			12907458	T	A	M229L	0.004
			12907469	C	G	S225T	4.529
			12907508	T	C	E212G	0.001
			12907518	T	A	S209C	8.642
			12907519	C	A	Q208H	7.204
TAS2R46	#3	12	11214509	C	T	V129I	0.007
	#5		11214299	T	C	I199V	6.334
	#7		11214264	C	G	Q210H	6.436
USH2A	#4	1	216373317	T	G	S1155R	15.81
	#5		216052233	G	A	P2811S	20.1
	#6		216591996	C	A	G171W	19.45

TAS2R43	#5	12	11244027	T	C	R268G	0.003
			11244036	T	G	K265Q	0.003
			11244067	A	ATT	S254R?	NA
			11244070	TCC	T	G253	NA
			11244091	C	T	M246I	0.004
			11244096	T	C	I245V	1.152
			11244102	G	C	L243V	2.621
			11244721	G	C	F36L	5.223
			11244723	A	C	F36V	0.001
			11244731	A	G	I33T	0.721
			11244797	C	A	S11I	0
	#6		11244721	G	C	F36L	5.223
			11244723	A	C	F36V	0.001
			11244731	A	G	I33T	0.721
	#7		11244199	C	G	Q210H	3.385
			11244721	G	C	F36L	5.223
			11244723	A	C	F36V	0.001
			11244731	A	G	I33T	0.721
GPR98	#1	5	90050870	G	C	L3816F	12.83
	#2		89933756	C	CT	S744S?	NA
			89933758	C	CTAAAATATATGTTCAGAAATTA	L745LKYMFRNY?	NA

Supplementary Table 4.1 continues on next page

Supplementary Table 4.1. ***Continued***

GENE	PATIENT	CHROM	POS	REF	ALT	AA CHANGE	CADD score
ZNF679	#1	7	63721279	G	C	K78N	5.195
	#2		63721287	A	G	E81G	5.972
ROBO2	#1	3	75986717	G	A	V25M	3.198
	#2		77600125	C	CAATATTCA	L406QYS?	NA
			77666794	G	T	A1142S	21
RNMT	#1	18	13731869	C	CAAACTCTTT	S118SNSF	NA
	#2		13731871	A	AACTCTTTTCT	T119NSFL?	NA
			13737126	T	TC	V224V?	NA
C4BPB	#1	1	207268780	G	A	S175N	0.247
	#2		207268780	G	A	S175N	0.247
			207268786	A	C	H177P	2.195
TEX15	#1	8	30702149	C	T	W1462*	39
	#2		30694422	C	CTTTATTGAGTTGTTA	R2743RNNSIK	NA
DOCK2	#1	5	169494534	G	C	E1496D	16.78
	#3		169507234	C	T	A1745V	6.065
ADAMTS2	#1	5	178585847	G	C	Q337E	28.6
	#4		178548685	G	T	S1052*	40
INVS	#1	9	103054721	G	A	E728K	17.01
	#4		103046637	A	G	E607G	13.24

ZNF880	#1	19	52877732	A	G	K107R	NA
	#4		52888074	G	GAT	G414G?	NA
CUL7	#1	6	43020271	C	T	G138S	22
	#4		43010692	C	T	D1249N	17.54
NHSL2	#1	X	71360567	C	G	Q1057E	0.034
	#4		71360022	C	T	P875L	18.31
CD55	#1	1	207520819	A	C	D370A	1.415
	#5		207523493	G	A	M364I	3.954
USP24	#1	1	55622999	C	A	K424N	14.69
	#5		55620115	C	T	V527I	16.94
VPS13A	#1	9	79934573	G	GTTTTTA	W1800CFL?	NA
	#5		79968357	GAC	G	T2485	NA
IL33	#1	9	6241703	TAAA	T	K4	NA
	#5		6256046	G	A	E231K	25.9
ADAMTS12	#1	5	33614463	T	C	N803D	27.8
	#6		33576452	TG	T	P1226	NA
TAS2R31	#1	12	11183837	A	G	I33T	5.251
	#6		11183697	C	A	V80L	2.153
			11183708	T	C	Y76C	5.935
			11183722	A	C	F71L	0.01

Supplementary Table 4.1 continues on next page

Supplementary Table 4.1. ***Continued***

GENE	PATIENT	CHROM	POS	REF	ALT	AA CHANGE	CADD score
TPSB2	#1	16	1279732	C	A	G23V	12.04
	#6		1279714	A	G	V29A	8.603
			1279717	C	T	R28Q	9.729
MUC12	#1	7	100616310	G	C	E91D	2.375
			100616313	T	A	S92R	2.387
			100616320	G	A	V95M	3.904
			100616336	G	A	W100*	11.98
			100616338	A	G	M101V	0.417
			100616344	G	A	G103R	2.979
	#7		100616227	A	T	R64W	3.244
			100616229	G	C	R64S	2.205
OR2T4	#1	1	248525100	G	A	G73E	8.963
			248525135	G	A	A85T	4.912
			248525138	C	T	H86Y	4.27
	#7		248525100	G	A	G73E	8.963
CNN2	#1	19	1037756	G	A	G284S	28.7
			1037766	G	A	R287Q	19.93
	#7		1037766	G	A	R287Q	19.93
CNKSR3	#2	6	154743741	G	T	P282T	20.4
	#3		154732140	C	A	D403Y	15.56

LAMA3	#2	18	21511146	G	T	V2853L	14.59
	#3		21453105	C	A	Q33K	24.1
EML6	#2	2	55056611	A	ATGCTAAAGATTCC	K282MLKIP?	NA
	#3		55040448	T	A	Y93N	21.6
TTN	#2	2	179556813	G	GTGTTTTTACTGTTT	P10564PNSKN?	NA
			179611064	C	A	V5355L	6.771
			179642503	C	A	E1470*	46
	#4		179428105	G	T	S27585Y	14.93
COL4A5	#2	X	107868992	T	A	L1025H	10.48
	#5		107834817	C	G	P456A	7.616
KNDC1	#2	10	134980924	C	T	R48C	11.02
	#5		135012509	C	T	R833*	40
EPB41	#2	1	29319962	T	A	L197I	11.9
			29319963	T	A	L197*	15.21
	#5		29438905	C	T	T814I	28.4
HLA-B	#2	6	31324144	T	G	Y140S	0.011
	#5		31324552	G	C	R86G	6.275
PKHD1L1	#2	8	110412514	A	ACC	I408T?	NA
	#6		110453081	G	A	G1367R	20.7
KLHL23	#2	2	170591981	G	GAGAGTTTTATTTTATTTTT	E153ESFILFL?	NA
	#7		170591937	A	G	N138S	20.8

Supplementary Table 4.1 continues on next page

Supplementary Table 4.1. ***Continued***

GENE	PATIENT	CHROM	POS	REF	ALT	AA CHANGE	CADD score
EVPL	#3	17	74011444	G	A	A653V	19.65
			74011445	C	A	A653S	34
	#4		74017617	ACTCCATCTTCAGGGCCTC	A	EALKME309	NA
FZR1	#3	19	3526379	T	TATTCCC	F128YSL	NA
	#4		3526374	G	GGC	G126G?	NA
			3526377	T	TTTAC	L127LY?	NA
			3526379	T	TATTCCC	F128YSL	NA
CACNA2D1	#3	7	81599215	T	A	T776S	11.77
	#5		81799909	G	A	A104V	28.1
CDC27	#3	17	45234406	CA	C	A238	NA
			45234417	A	G	I235T	16.6
	#5		45214690	G	A	R587W	21.7
KCNT2	#3	1	196309693	C	T	V521I	16.76
	#6		196274404	G	T	A852D	23.7
KIAA1468	#3	18	59931309	T	G	L813R	17.37
	#6		59895774	G	A	G464E	0.011
EMP2	#3	16	10626861	T	TTG	R135S?	NA
			10631869	C	A	A78S	5.93
			10631877	C	G	C75S	5.486
	#7		10631877	C	G	C75S	5.486

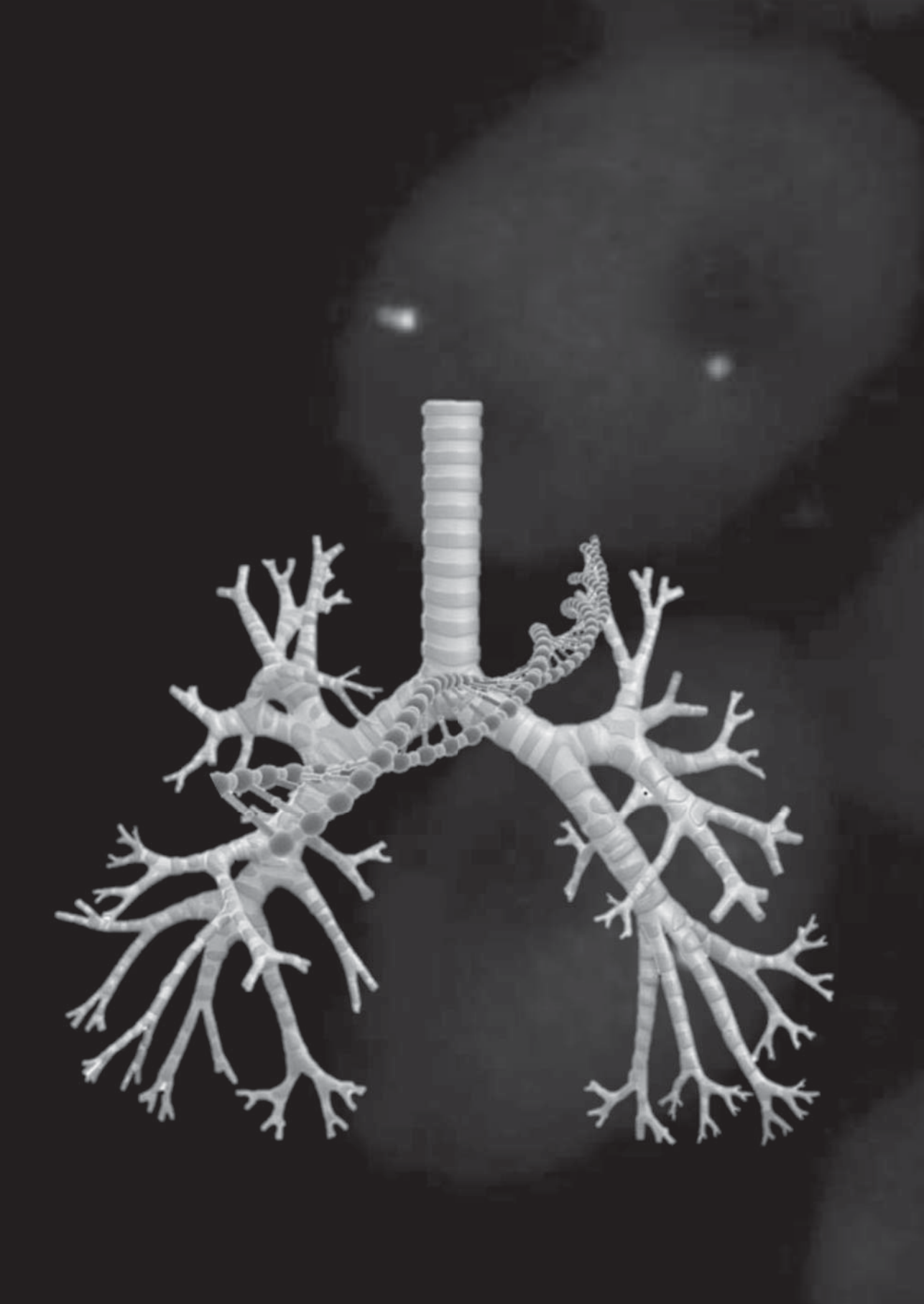
OR4C5	#4	11	48387285	A	AGTCTTTAGTAG	C244CLLK?	NA
	#5		48387155	T	C	Y288C	2.41
			48387930	T	C	I30V	0.004
			48387934	G	T	N28K	6.371
			48387939	C	G	V27L	9.711
			48387945	C	CCA	F24F?	NA
			48387946	G	T	F24L	2.36
MST1	#4	3	49723321	AGCGCTG	A	QR406	NA
	#5		49723321	AGCGCTG	A	QR406	NA
ARHGAP32	#4	11	128963571	A	T	M145K	12.46
			128963569	TC	T	M145	NA
	#5		128840090	G	A	S1659F	16.15
NLGN4X	#4	X	5811532	G	A	L613F	15.4
	#5		5811532	G	A	L613F	15.4
ZNF611	#4	19	53208749	C	G	S520T	1.295
	#6		53208521	C	G	C596S	15.97
HLA-DQA1	#4	6	32609207	C	T	A68V	0.008
			32609254	G	T	G84C	9.414
			32609255	G	T	G84V	7.745
			32609873	G	C	Q152H	0.005
	#7		32609254	G	T	G84C	9.414
			32609255	G	T	G84V	7.745

Supplementary Table 4.1 continues on next page

Supplementary Table 4.1. ***Continued***

GENE	PATIENT	CHROM	POS	REF	ALT	AA CHANGE	CADD score
HERC2	#5	15	28518112	C	A	S280I	13.15
			28518114	TC	T	G279	NA
			28518130	G	C	A274G	2.432
	#6		28518114	TC	T	G279	NA
			28518130	G	C	A274G	2.432
			28518136	G	A	T272M	7.967
ASTN2	#6	9	119903716	G	A	Q353*	38
	#7		119903623	G	A	R384W	14.32
MROH2B	#6	5	41067220	C	T	R64K	21.9
	#7		41061811	C	T	S159N	19.96
SP8	#6	7	20824778	C	A	V220L	12.45
	#7		20825145	GGCAGCCGCGGCTGCTGCCGCGGCCGCC	G	AAAAAAAAAA88A	NA
PRSS1	#6	7	142460764	G	A	V227I	0
	#7		142458526	A	G	N54S	0.009
			142458527	C	G	N54K	9.055
			142458531	C	T	Q56*	13.15

chrom, chromosome; pos, bp location; ref/alt, reference and altered nucleotides at mutated position; AA change, amino acid change; CADD, Combined Annotation Dependent Depletion score; CADD score ≥10 indicates a position within the top 10% most deleterious mutations. A score of ≥20 indicates a position within the top 1% most deleterious mutations.



5

Mutations in EMT related genes are an ALK independent resistance mechanism induced by crizotinib in ALK positive non-small cell lung cancer

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Submitted

Abstract

Background

Patients with ALK rearrangement positive lung cancer should receive crizotinib as the first line treatment. Although effective, most tumours develop resistance and show disease progression within one year, while a minority of patients do not respond at all. Mechanisms underlying resistance are only partly understood and studies on paired pre- and post-therapy biopsies are missing.

Methods

From our cohort of 29 ALK positive patients with advanced non-squamous NSCLC, we were able to retrieve sufficient tumour tissue from four responders before crizotinib treatment and upon resistance. For one non-responding patient, tumour tissue was obtained only before start of treatment. Somatic variants were detected by whole exome sequencing (WES) and pathway analysis was performed on resistant specific mutated genes in crizotinib-resistant samples.

Results

We identified 583 somatic mutations in crizotinib-resistant tumours, among which 137 mutations in 126 genes are resistant specific. These 126 genes were significantly enriched in 14 pathways, of which 9 genes related to the proteoglycans in cancer, HIF-1 signalling pathway, ECM-receptor interaction pathways, adherens junction, which are all related to epithelial-mesenchymal transition (EMT). Analysis of all EMT related pathways revealed 3 additional genes enriched in resistant tumours.

Conclusion

We observed a clear enrichment of mutations in genes associated with EMT related pathways, indicating that EMT may represent an important crizotinib resistance mechanism.

1. Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide[1]. Based on the traditional classification, there are two major types of lung cancer, small cell lung cancer and non-small cell lung cancer (NSCLC). NSCLC is divided into squamous, adenocarcinoma and large cell carcinoma, of which adenocarcinoma is the most common subtype. During the last decade, the clinical management and treatment of lung cancer patients has become more dependent on the molecular classification defined by 'driver' mutations that occur in genes like ALK, EGFR and ROS1. About 5% of all adenocarcinomas have a break in the ALK gene, resulting in a fusion product containing the kinase domain of the ALK protein fused to EML4 or other fusion proteins[2,3]. Tumour cells with an ALK-break are highly sensitive to tyrosine kinase inhibitors (TKIs) targeting ALK, such as crizotinib, or second-generation ALK inhibitors, ceritinib and alectinib. However, most responders will inevitably acquire resistance against the TKI treatment within one year[4,5].

The mechanisms of resistance can be divided into ALK-dependent and ALK-independent[6]. Approximately one third of the patients seem to have ALK-dependent resistant mechanisms, which include secondary ALK mutations and amplifications. Several ALK-independent mechanisms have been proposed based on studies in post-crizotinib tumour samples and studies in cell line models[7,8]. These mechanisms include mutations in and amplifications of EGFR, KIT, MET and IGFR-1R, as well as activation of the MAP kinase pathway, PI3K/AKT pathways, Ras/MAPK pathways and JAK/STAT pathways[9-13]. However, the level of proof is highly variable and the complete picture of all mechanisms involved in crizotinib resistance is not clear yet.

In this study, we sought to further characterize crizotinib treatment resistant-related somatic mutations using whole exome sequencing (WES) of paired tumour biopsies of advanced adenocarcinoma patients before and after crizotinib treatment.

2. Methods

2.1. Patient selection

In our previous study, we described 29 non-squamous NSCLC patients who were ALK break positive and were treated with crizotinib[14]. For four out of 29 patients, we had sufficient pre- and post-treatment tumour samples (formalin fixed paraffin embedded (FFPE) or frozen) for WES in this study. For one non-responding patient we had sufficient FFPE material for

WES. The patients are numbered as ALK4, ALK6, ALK8, ALK14, and ALK16 in concordance with our previous study. ALK4 was shown to have an ALK G1269A mutation based on our previously reported RNA-seq data[15]. White blood cells were isolated from peripheral venous blood and used as normal control. Tumour samples before crizotinib treatment from patient ALK15 and upon resistance to crizotinib treatment from patients ALK4 and ALK6 were fresh frozen, whereas the other tumour specimens were FFPE. Macro dissection was applied to the tumour samples to achieve a tumour cell content of more than 60%.

2.2. DNA isolation

DNA from blood and frozen samples was isolated using a standard salt-chloroform DNA isolation protocol. For FFPE samples, DNA was isolated using the ReliaPrep™ FFPE gDNA Miniprep System kit (Promega, Madison, USA) following protocol of the manufacturer. The concentrations of the DNA samples were measured by NanoDrop (Thermo Fisher Scientific Inc., Waltham, USA), and the quality was evaluated on a 1% agarose gel.

2.3. Whole exome sequencing

WES was carried out by BGI (BGI Tech Solutions Co. Ltd, Hong Kong) on 0.6-2µg genomic DNA of normal and tumour derived DNA samples. Target enrichment was done using the Agilent SureSelect All Exon V5 kit (Agilent technologies, Santa Clara, USA). Paired-end sequencing with a read length of 2x100nt was performed on Illumina HiSeq2000. As part of the validation procedure we performed a second WES on the crizotinib resistant samples of patients ALK4 and ALK6 using the protocol and data analysis pipeline as published previously[15]. Also, RNA-sequencing data of resistant samples of ALK4 and ALK6 were re-analysed to confirm presence of mutations detected in the BGI data[15].

2.4. Bioinformatics approach

The variant calling pipeline is an adaption of the GATK workflow and molgenis compute as workflow management software[16-18]. Alignment of reads was done using BWA and the Genome Analysis Toolkit (GATK), using the human genome reference build GRCH37 with decoys from the GATK bundle[19]. Picard Tools were used for format conversion and marking duplicates. As variant caller, this pipeline uses the HaplotypeCaller for all the samples of the same patient/cohort. Variants were annotated using SnpEff / SnpSift with the ensembl release 75 gene annotations and the dbNSFP2.0 database[20-22]. GATK was used to

identify variant annotated in dbsnp 138, Cosmic v72, 1000 genomes phase 3 and Exac 0.3 databases[23-26]. The data were filtered for quality metrics similar to GATK recommendations and custom filters for population frequency and variant effect.

Combined Annotation Dependent Depletion (CADD) scores were used to predict pathogenicity of the identified variants. Variants with a CADD score equal or more than twenty are defined as deleterious; those between 10 and 20 are possibly deleterious and those below 10 are non-deleterious[27].

To identify somatic mutations we first excluded variants for which the total number of reads in the normal sample was less than ten. Next, we excluded all variants for which one or more mutant reads were present in the normal sample. Then, variants with total reads less than ten in either the primary or resistant samples were excluded. The remaining variants with two or more mutant reads in either the pre-treatment tumour samples or post-treatment tumour samples were considered as somatic.

Variants with mutant read frequencies (MRFs) ≥20% in resistant samples, and MRFs in the resistant sample at least two times more than those in the paired primary sample were marked as "treatment-related" variants. Pathway analysis was performed with Partek Genomics Suite 6.6 (Partek Inc., St Louis, MO) software using all genes with crizotinib resistance-related variants.

2.5. WES-based copy number variant (CNV) analysis

Pseudo probe data were generated with SAMtools, VarScan2 and DNAcopy[28-30]. Briefly, for each sample the pseudo probe derived GC-normalized log2 copy number ratios were generated using the corresponding normal sample in case frozen tumour tissue was used for isolation of high quality DNA samples. For tumour samples with low quality DNA (all FFPE sample) we used WES data of a merged pool of normal samples as the reference. All alignments with a mapping quality greater than 40 in combination with a minimal segment size of 2kb and a maximal segment size of 5kb with a mean base-wise coverage of at least one were used to calculate the ratios. CNV plots of the resistant tumour sample were compared to the CNV plot of the paired primary tumour sample by a combination of the calculated ratios in identical bins based on changes in copy number level and by visual inspection.

3. Results

3.1. Patients

Four patients (ALK4, ALK6, ALK14, and ALK16) developed resistance to crizotinib after an initial response of approximately one year (Table 5.1 and Figure 5.1). One patient, ALK8, had no tumour response on crizotinib, and died after one month. ALK6 and ALK16 deceased 15 months and 3 years after end of crizotinib treatment, respectively. ALK4 and ALK14 patients are still alive at 12 and 9 years after initial diagnosis, respectively.

Table 5.1. Characteristics of five ALK rearranged advanced NSCLC patients

					Sample Location	
Patient	Gender	Age at diagnosis	PFS* (m)	Smoking status (PY**)	Primary tumour biopsy	Resistant tumour biopsy
ALK4	F	34	15.9	Non-smoker	Ovary	Liver
ALK6	M	55	9.5	Past-smoker (15)	Cervical lymph node	Glenoid
ALK8	M	76	1.6	Past-smoker (NA)	Lymph node mediastinal (4R)	ND
ALK14	F	62	8.4	Current smoker (20)	Brain occipital metastasis	Mediastinal lymph node (7)
ALK16	F	48	4.1	Past-smoker (18)	Bronchial biopsy	Lung

*PFS: progression free (time from start of treatment to the examination by CT scan where we have seen progressive disease or death). **PY: pack years. ND: not done.

3.2. Whole exome sequencing and its validation

WES generated an average of 60 x 10^6 unique reads that passed the Illumina quality filtering steps per sample (Supplementary Table 5.1). An average of 98% of the unique reads could be aligned to the human reference genome. The mean coverage per sample was 66x with 86% of the target region covered at least 20x.

We identified 583 variants in 519 genes across the five patients. Among these 583 variants, 20% (116 SNVs in 143 genes) had CADD scores equal or higher than 20; 45% (265 SNVs in 253 genes) had CADD scores between 10 and 20; 26% (151 SNVs in 101 genes) had CADD scores less than 10. For 9% of the variants (51 variants in 47 genes) no CADD scores were available; 47/51 variants were small insertions or deletions (INDELs).

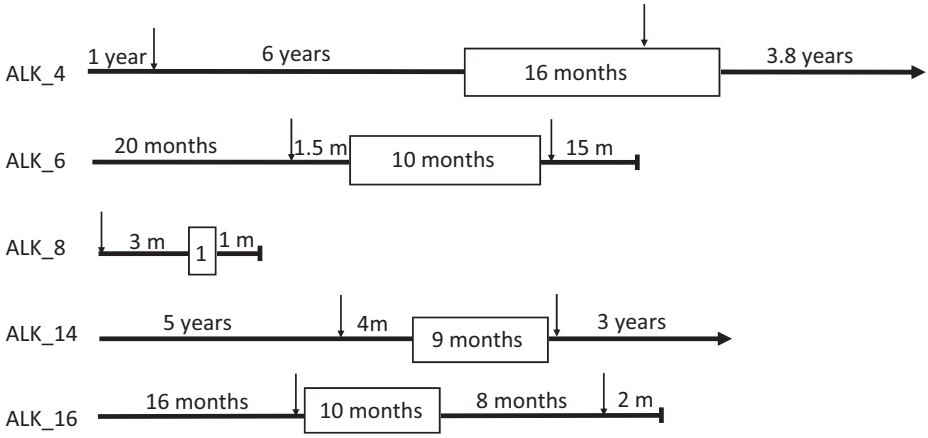

Figure 5.1. Timelines of crizotinib treatment period and pre- and post-crizotinib tissue collection.
The arrow indicates the time of biopsy. The block indicates the duration of crizotinib treatment. Timelines are provided from the diagnosis to the first biopsy, from the second biopsy to end of the study or death. Of note, when the second biopsy was taken in patient ALK4, tumour relapse had been observed for which local treatment was given and treatment was continued. Patient ALK16 has been treated beyond progression.

We analysed validity of the WES data by two independent approaches. We first re-analysed the RNA-seq data from our published study, which included two crizotinib-resistant tumour samples of ALK4 and ALK6 patients[15]. A coverage of ≥10x used as a criterion to allow a reliable confirmation was observed for 95 out of the 169 variants in ALK4 and 34 out of the 61 variants in ALK6; 90 out of the 95 variants (95%) in ALK4 and 29 out of 34 (85%) in ALK6 mutant reads were observed consistent with those identified in the WES data, indicating that the vast majority of the mutated genes were expressed in these tumour samples. The second approach was based on an independent WES, again focusing on variants with a coverage of ≥10x. Out of the 67 variants in ALK4 and the 42 variants in ALK6, we confirmed 61 (91%) variants in ALK4 and 40 (95%) variants in ALK 6. In total, we independently validated 114 out of 123 (93%) variants in ALK 4 and 45 out of 48 (94%) variants in ALK6.

3.3. Crizotinib treatment-related variants

A comparison of the primary to the resistant tumour in all four paired patients revealed in total 176 different "treatment-related" variants in 156 genes (Figure 5.2). Among these variants 16% (21 SNVs in 15 genes) had CADD scores above 20, 43% (75 SNVs in 71 genes) had CADD scores between 10 and 20, 30% (53 SNVs in 49 genes) had CADD scores less than 10 and 11% (20 INDELs in 19 genes) had no CADD scores. The distribution of the variants

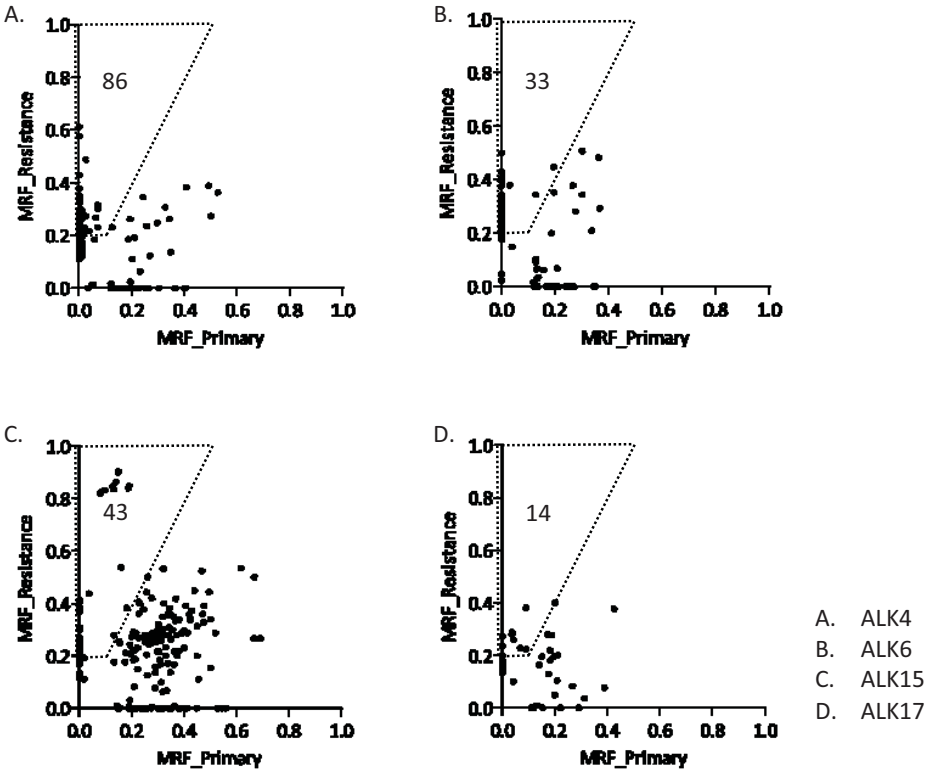

Figure 5.2. Comparison of mutant read frequencies (MRF) in primary (x-axis) and resistant samples (y-axis). Each spot is a single variant. Variants with mutant read frequencies (MRFs) ≥20% in resistant samples and MRFs in the resistant sample at least two times more than those in the paired primary sample (number in figure) were marked as "treatment-related" variants.

over the different CADD score groups was similar to the distribution of all somatic variants. One gene, BZRAP1, was mutated in more than one patient only. The 156 mutated genes were present in 167 pathways. A significant enrichment was observed for 20 pathways. To focus on ALK-independent resistance mechanisms, we next excluded gate keeper genes mutated in patient ALK4, which was known to carry an ALK mutation that explains the crizotinib resistance. The three paired patients had a total of 90 "treatment-related" variants in 74 genes. All genes were mutated only in one of the three patients. These 74 genes were present in 105 pathways of whom 15 pathways were significantly enriched (Supplementary Table 5.2). Five of these were related to "hormonal pathways", three to "specific cancer subtypes", one to "hepatitis B", one to "GABAergic synapse" and one to "arrhythmogenic right ventricular cardiomyopathy".

Four of the pathways including 9 genes were linked to EMT, i.e. proteoglycans in cancer, HIF-1 signalling pathway, FoxO signalling pathway and ECM-receptor interaction (Table 5.2). Four of the genes, i.e. ARNT, PTPN11, SMAD4, VEGFA, variants had CADD scores of more than 20, whereas no CADD scores was available for one gene, i.e. LAMA2, having an out of frame INDEL (LAMA2 has two nucleotides deleted).

In ALK8, the patient without a response to crizotinib, somatic variants in three EMT related genes, i.e. ITGAM, CACNA1E, and RUVBL1, were observed. These three genes were involved in the cell adhesion molecules (CAMs), MAPK signalling, Wnt signalling, and regulation of actin cytoskeleton. Pathway analysis per patient on genes with treatment-related variants revealed only one pathway that was shared by all five patients, i.e. metabolism pathway.

Table 5.2. EMT related significantly enriched pathways based on crizotinib induced mutated genes in patients ALK6, ALK14 and ALK16

Pathway name	Genes mutated	Enrichment score	Enrichment p-value	Genes mutated / genes not mutated
Proteoglycans in cancer	ANK2, FASLG, HSPG2, PTPN11, STAT3, VEGFA	8.0	0.00034	6/1049
HIF-1 signalling	ARNT, STAT3, VEGFA	4.3	0.01391	3/548
FoxO signalling	FASLG, SMAD4, STAT3	3.7	0.02598	3/696
ECM-receptor interaction	HSPG2, LAMA2	3.3	0.03764	2/331

3.4. Treatment-related CNVs

We compared WES-based CNV plots of the primary tumours to the paired resistant samples. Although we did identify several differences in copy number variations between primary and resistant samples (Figure 5.3), we did not see amplification of the ALK, MET or KIT gene loci known to be associated with resistance in any of the patients. Moreover, no recurrent CNVs were observed that were shared between the 4 patients in other parts of the genome.

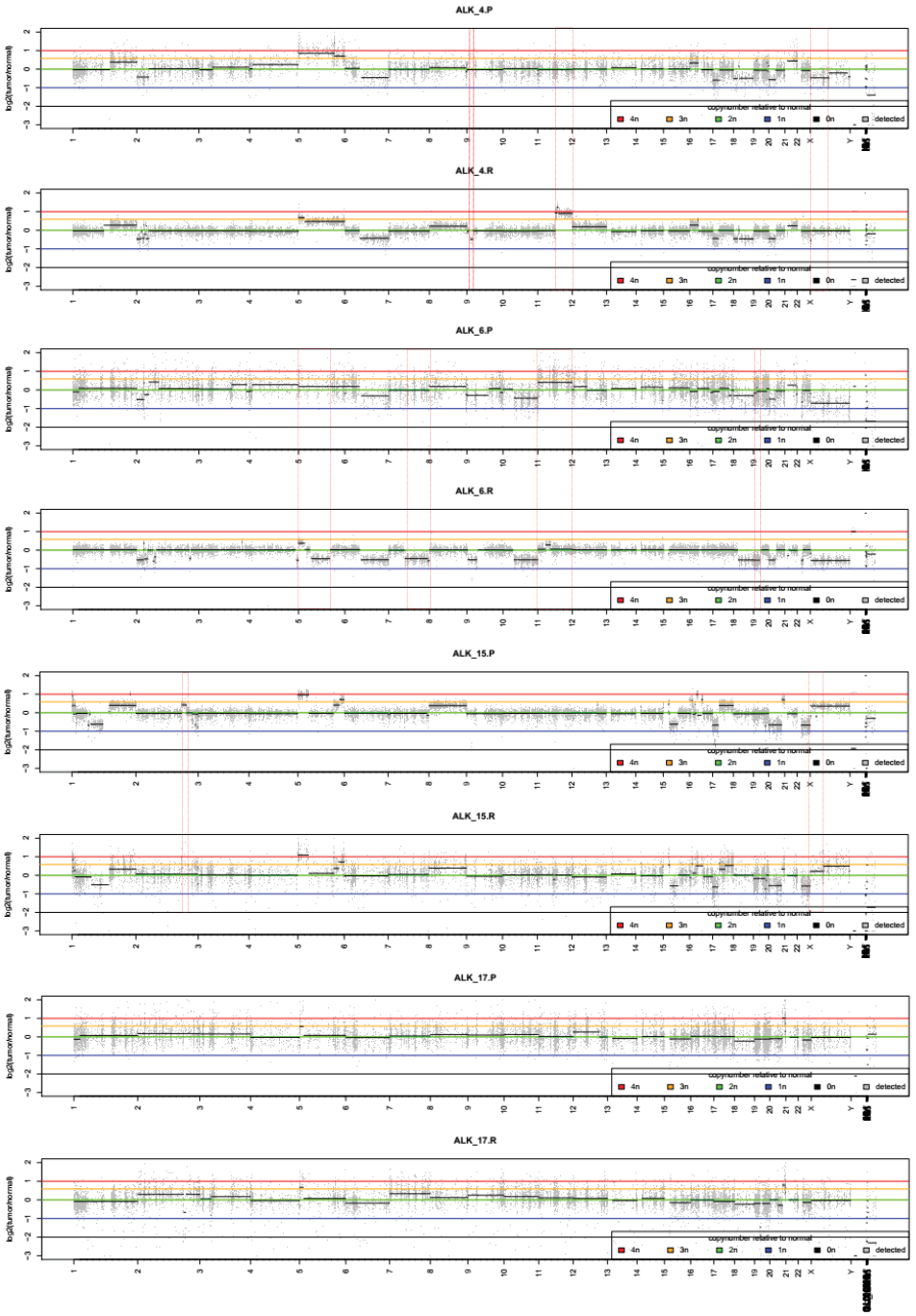

Figure 5.3. CNV plots across four patients with ALK rearranged advanced NSCLC with matched primary and resistant tumour samples.

The chromosomes with conspicuous changes between primary and resistant samples in each patient are marked in red box.

4. Discussion

In this study, we identified 176 variants in 156 genes that are specific for or more enriched in crizotinib resistant samples as compared with the matched tumour sample before crizotinib treatment. Pathways analysis based on genes with treatment related mutations revealed a significant enrichment of 9 genes in four pathways, i.e. proteoglycans in cancer (ANK2, FASLG, HSPG2, PTPN11, STAT3, VEGFA), HIF-1 signalling pathway (ARNT, STAT3, VEGFA), FoxO signalling pathway (FASLG, SMAD4, STAT3), and ECM-receptor (extracellular matrix) interaction pathway (HSPG2, LAMA2). These four pathways are all involved in EMT and gene mutations were found in two of the three patients with ALK-independent resistance mechanisms.

To our knowledge, we are the first to explore ALK-independent mechanisms of crizotinib resistance in advanced NSCLC patients by comparing variants observed in the crizotinib resistant tumour to the variants present in the tumour sample before crizotinib treatment. Many crizotinib resistance mechanisms have been proposed based on patients samples and cell line studies. However, these studies were performed on resistant samples without comparison to the pre-treatment samples. For example, activation of ALK-independent bypass mechanisms, such as activation of EGFR, KRAS, SRC and MAPK signaling, were shown in resistant samples from NSCLC patients[8,32,33]. Although these studies generated valuable data, it does not allow to pinpoint the true treatment induced alterations. Although the total number of matched samples was still quite limited, we present the first comparative analysis on pre- and post-crizotinib treatment tumour specimens in ALK+ NSCLC patients. A main problem in lung cancer patients is the scarceness of re-biopsies, which generally are relatively small and with low tumour cell percentages. These biopsies are frequently used in total for the initial diagnostic tests to guide further therapy. In future studies, we may be able to use liquid-biopsy based material, which will be easier to obtain and will allow with rapid improvements in NGS techniques genome wide analysis.

The limited DNA quality of the FFPE tumour samples resulted in a suboptimal coverage and this precluded detection of mutations in sub clones of the tumour. Although such coverage might have resulted in an underestimation of somatic variants, it should not have affected the identification of treatment-related mutations because of the use of resistant tumour samples. Nevertheless, we also experienced limited biopsy sizes and therefore extensive validations of the observed variants was limited.

Based on previous studies, the resistant mechanisms against ALK inhibitors can be classified into ALK-dependent and ALK-independent mechanisms[10]. Like many other studies, we

found the known *ALK* G1269A mutation present in the resistant tumour while absent in the primary tumour in patient ALK4, which is in accordance with the RNA-seq result in our previous and other studies[7,15,34,35].

For the remaining three patients, no ALK gate keeper mutations were found, indicating possible activation of ALK-independent bypass mechanisms. Since there was no overlap between the genes mutated in these three patients, it might be that ALK-independent mechanisms are quite diverse. This would be consistent with the broad variation in resistance mechanisms proposed in the literature[9,34]. To find a possible common mode of action, we proceeded with pathway analysis on the potentially treatment-related genes in these three patients. Among the significantly enriched pathways, we found four EMT related pathways and the subsequent analysis of all other EMT related pathways revealed a total of 12 mutated genes that are involved in EMT-related pathways. Several studies either on cell lines studies have proven the potential role of EMT as a mediator of resistance against ALK inhibitors. Silencing of vimentin restored the responsiveness of ALK break positive, crizotinib-resistant cells to ALK inhibitors, indicating a causal relation between EMT and crizotinib resistance[36]. Five out of eleven *ALK*+ NSCLC patients who were treated with the second-generation ALK inhibitor ceritinib, showed EMT based on immunostaining for E-cadherin and vimentin[7]. In our study, seven of the 14 genes involved in EMT had variants with a potential impact on the protein based on a CADD score of more than 20 or presence of out of frame INDELs.

SMAD4 has been proven to be one of the most crucial genes in regulating the TGF-beta signalling pathway. The TGF-beta pathway is one of the most important pathways leading to EMT by e.g. TGF-beta-SMAD signalling, induction of microRNA expression, decreasing expression of epithelial splicing regulatory proteins (ESRPs), PI3K–AKT–mTORC1 signalling, and cytoskeletal changes[37]. HIF-1 has been shown to activate the expression of SNAI1 by binding to two hypoxia response elements (HREs) in the promoter. SNAI1 represses expression of the epithelial marker E-cadherin and enhances expression of the mesenchymal markers β-catenin and vimentin[38,39]. By knockdown and/or transfection experiments on cell line studies related to several cancer types including prostate cancer, gastric cancer and breast cancer, three out of the four FOXO family members (FOXO1, FOXO3a, FOXO4, except for FOXO6) have been proven to regulate EMT by repressing E-cadherin expression and promoting SNAIL expression[40-42]. Some proteoglycans like syndecans are required to maintain the epithelial characteristic of basolateral surfaces and within adhesive junctions. Deficiencies in cell surface syndecan-1 lead to markedly reduced E-cadherin expression in

normal gland epithelia[43,44]. ECM has been shown to promote EMT by weakening cell-cell adhesions[37,45].

The only recurrently mutated gene in more than one crizotinib resistant patients is BZRAP1, which is a synaptic transmission regulator. This gene has never been described in cancer related resistance.

In case of crizotinib resistance, there are second and third generation ALK inhibitors such as ceritinib, alectinib, brigatinib and lorlatinib that can be used as next treatment option[46]. These drugs will be effective especially for ALK dependent resistant mechanism, based on their improved binding to the ATP binding site. It is still unclear whether these drugs also show tumour response in ALK independent resistant mechanism. This should be a further focus of future clinical trials in patients with ALK-independent crizotinib-resistance mechanisms.

In conclusion, the most significant finding in this study is the identification of mutations in genes involved in EMT-related pathways. From a clinical perspective, the mutational status of patients may provide therapeutic guidance for clinical management and the future use of EMT blocking agents in NSCLC patients should be studied further. This could therefore be a new target for treatment.

5. References

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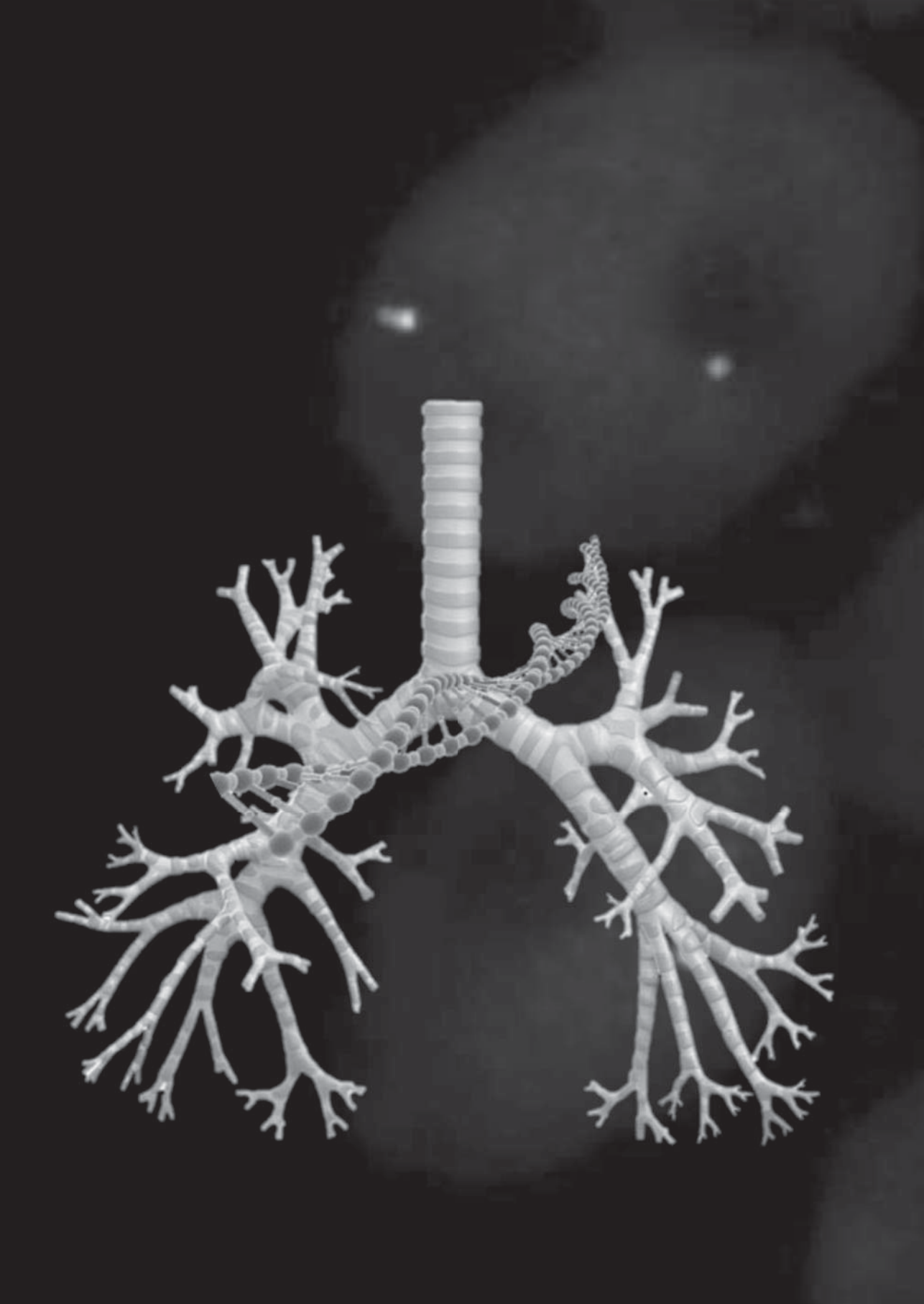
Supplementary Table 5.1. EMT related significantly enriched pathways based on crizotinib induced mutated genes in patients

Sample	ALK4			ALK6			ALK8		ALK14			ALK16		
	N	T	R	N	T	R	N	R	N	T	R	N	T	R
Clean reads x10^6	61	74	64	60	66	65	58	59	59	58	57	61	142	61
Unique reads x10^6	58	62	60	57	59	60	57	58	56	56	55	58	82	60
Percentage of unique reads	95%	84%	94%	95%	89%	92%	97%	97%	96%	97%	96%	96%	58%	98%
Unique aligned reads (x10^6)	57	60	59	56	58	58	56	56	55	55	54	57	79	59
Percentage of unique aligned reads	98%	97%	98%	98%	98%	98%	98%	98%	98%	98%	98%	98%	96%	98%
Average target coverage	68	70	66	65	71	68	65	65	68	65	65	65	63	66
20X coverage	91%	90%	91%	91%	90%	91%	90%	65%	91%	89%	74%	91%	91%	73%
Number of variants	0	82	169	0	44	61	0	31	0	168	180	0	29	38
CADD scores														
<10	0	20	28	0	13	15	0	5	0	34	45	0	4	6
[10,20)	0	34	89	0	17	23	0	16	0	77	77	0	14	14
≥20	0	18	46	0	8	13	0	6	0	50	46	0	7	9
NA	0	10	6	0	6	10	0	4	0	7	12	0	4	9

N: normal sample; T: tumour sample before crizotinib treatment; R: tumour sample when resistance was observed either during or after crizotinib treatment. CADD: Combined Annotation Dependent Depletion scores used to predict pathogenicity of the identified variants.

Supplementary Table 5.2. Significantly enriched pathways harboring treatment-related variants in ALK6, ALK14, and ALK16

Pathway name	Genes mutated	Enrichment score	Enrichment p-value	Genes mutated / genes not mutated
Proteoglycans in cancer	ANK2, FASLG, HSPG2, PTPN11, STAT3, VEGFA	8	0.00034	6/1049
Pathways in cancer	ARNT, CTNNA3, FASLG, LAMA2, SMAD4, STAT3, VEGFA	6.5	0.00154	7/1932
AGE-RAGE signaling pathway in diabetic complications	PLCD1, SMAD4, STAT3, VEGFA	6.4	0.00169	4/560
Renal cell carcinoma	ARNT, PTPN11, VEGFA	5.4	0.00455	3/363
Hepatitis B	FASLG, HSPG2, SMAD4, STAT3	5.3	0.00489	4/754
Pancreatic cancer	SMAD4, STAT3, VEGFA	5	0.00662	3/416
HIF-1 signaling pathway	ARNT, STAT3, VEGFA	4.3	0.01391	3/548
Type I diabetes mellitus	FASLG, GAD2	4.2	0.01474	2/199
FoxO signaling pathway	FASLG, SMAD4, STAT3	3.7	0.02598	3/696
GABAergic synapse	GAD2, SLC6A1	3.4	0.03323	2/309
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	CTNNA3, LAMA2	3.3	0.03601	2/323
Taurine and hypotaurine metabolism	GAD2	3.3	0.0363	1/1939
ECM-receptor interaction	HSPG2, LAMA2	3.3	0.03764	2/331
Aldosterone synthesis and secretion	DAGLA, SCARB1	3.1	0.04574	2/369
Adipocytokine signaling pathway	PTPN11, STAT3	3.1	0.04708	2/375



6

Genomic aberrations in crizotinib resistant lung adenocarcinoma samples identified by transcriptome sequencing

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Abstract

ALK-break positive non-small cell lung cancer (NSCLC) patients initially respond to crizotinib, but resistance occurs inevitably. In this study we aimed to identify fusion genes in crizotinib resistant tumour samples. Re-biopsies of three patients were subjected to paired-end RNA sequencing to identify fusion genes using deFuse and EricScript. The IGV browser was used to determine presence of known resistance-associated mutations. Sanger sequencing was used to validate fusion genes and digital droplet PCR to validate mutations. *ALK* fusion genes were detected in all three patients with *EML4* being the fusion partner. One patient had no additional fusion genes. Another patient had one additional fusion gene, but without a predicted open reading frame (ORF). The third patient had three additional fusion genes, of which two were derived from the same chromosomal region as the *EML4-ALK*. A predicted ORF was identified only in the *CLIP4-VSNL1* fusion product. The fusion genes validated in the post-treatment sample were also present in the biopsy before crizotinib. *ALK* mutations (p.C1156Y and p.G1269A) detected in the re-biopsies of two patients, were not detected in pre-treatment biopsies. In conclusion, fusion genes identified in our study are unlikely to be involved in crizotinib resistance based on presence in pre-treatment biopsies. The detection of *ALK* mutations in post-treatment tumour samples of two patients underlines their role in crizotinib resistance.

1. Introduction

Chromosomal rearrangements involving the anaplastic lymphoma kinase (*ALK*) gene can occur in different cancers including NSCLC, anaplastic large cell lymphoma and inflammatory myofibroblastic tumors[1]. The echinoderm microtubule-associated protein-like 4 (EML4) gene is the most common fusion partner of the *ALK* gene in NSCLC[2]. Presence of an *EML4-ALK* fusion gene in NSCLC has been reported for the first time in 2007[3]. In addition, *KIF5B*, *KLC1* and *TFG* have also been described as fusion partners[4]. Injection of *EML4-ALK* overexpressing 3T3 cells into nude mice induced tumour growth indicating transforming activity of the EML-ALK fusion protein[3]. *ALK* rearrangements have been detected in approximately 4-7% of the NSCLC patients[3,5]. The frequency is higher in young, non-smoking patients with adenocarcinoma[6]. The *EML4-ALK* fusion results in overexpression of the fusion product that includes the tyrosine kinase activity domain of *ALK*[7].

Despite an initial favourable response to crizotinib, patients inevitably acquire resistance due to selective pressure of the tyrosine kinase inhibitor (TKI)[1]. Different genomic aberrations have been identified as resistance mechanisms to ALK-TKI, including ALK-dependent and ALK-independent mechanisms. ALK-dependent mechanisms include gatekeeper (L1196M) or other mutations such as C1156Y and G1269A in the *ALK* kinase domain and ALK copy number gain[8,9]. Gatekeeper mutations are defined as mutations in the gatekeeper residue of the tyrosine kinase protein, i.e. the leucine residue at position 1196[8]. ALK-independent mechanisms include *KRAS* and *EGFR* mutations (L858R), and *KIT* amplification. In addition, *AXL* overexpression and changes in the pathways of the epithelial-mesenchymal transition (EMT) have been described as a resistance mechanisms to the ALK-TKI in cell lines[10]. Despite the increasing number of known resistance mechanisms, the mechanisms remains unknown in approximately 18-44% of the patients[1,9].

As it is known that TKs can be activated by chromosomal translocations, we speculate that fusion genes might form a potential novel resistance mechanism. In this study we aimed to identify presence of fusion genes as a novel resistance mechanism in patients progressing on crizotinib using transcriptome sequencing. We used deFuse and EricScript to detect fusion genes in paired-end RNA sequencing (RNA-seq) data and validated fusion genes by RT-PCR and Sanger sequencing. Fusion genes confirmed in post-treatment samples were subsequently analysed in the pre-treatment samples. In addition, we used the RNA sequencing data to determine presence of crizotinib resistance-associated mutations in *EGFR*, *KRAS* and *ALK* genes.

2. Materials and methods

2.1. Patients and tumour samples

Patients were selected at our outpatient clinic of the University Medical Centre Groningen when they had non-squamous cell lung cancer with an *ALK* break as determined by FISH (> 15% breaks). Among 29 *ALK*-positive NSCLC patients treated between 2010 and 2013, we had frozen tissue available of crizotinib-resistant post-treatment tumour samples of three lung adenocarcinoma patients (Table 6.1). Formalin fixed paraffin embedded (FFPE) tumour tissue was available before and after crizotinib treatment for all three patients. A normal lung tissue sample was used as control for the RT-PCR.

2.2. Informed consent and ethics

Written informed consent for tumour tissue from all three patients was obtained before biobanking and retrieval from the Groningen Pathology biobank. All patient data were anonymised and de-identified prior to analysis (Table 6.1). The authors were not informed about identification variables. The study was approved by the Medical Ethical Committee of the University Medical Centre Groningen and conducted in accordance with the provisions of the Declaration of Helsinki and Good Clinical Practice guidelines. Due to the retrospective nature of this study, under Dutch Law for human medical research (WMO), no specific permission was compulsory from the Institutional Review Board.

2.3. Fluorescence in situ hybridization (FISH)

FISH was performed using the ALK dual colour break probes (Vysis LSI ALK Break Apart FISH Probe Kit, Abbott Molecular Inc., Des Plaines, USA) and *EML4-ALK* fusion FISH (Kreatech, Leica Biosystems, Wetzlar, Germany) following standard protocols. After deparaffinization, slides were incubated in TRIS/EDTA pH9.0 buffer in a pressure cooker for 7 min at 120°C. This was followed by an RNase (Thermo Fisher Scientific Inc., Waltham, USA) treatment step for 10 min at 37°C, followed by a pepsin (Sigma-Aldrich, St. Louis, United States) treatment for 1h at 37°C. Hybridization and wash steps were performed according the manufacturer´s protocol. Slides were mounted in vectashield with DAPI (1:1 diluted in vectashield). Three images were captured from each slide using an appropriate single filter (Olympus DP50 camera, USA). Scoring was performed according to the international guidelines (www.Abbott.com) by two independent well-trained and experienced readers and a case was

called ALK-break positive if ≥15% of the evaluated neoplastic nuclei (n=100) had a break-apart pattern. For the *EML4-ALK* fusion a case was called positive when >15% of the cells showed co-localization of the two FISH signals.

2.4. ALK immunohistochemistry

ALK immunohistochemistry (IHC) was performed on 3 micron FFPE tumour tissue sections, using the ALK rabbit monoclonal antibody clone D5F3 (Roche, Basel, Switzerland) in the VENTANA BenchMark ULTRA according to the manufacturer's protocol (Ventana, Tucson, Arizona). The staining was visualized using the OptiView DAB IHC Detection Kit (Ventana) and OptiView Amplification Kit (Ventana). Samples were scored ALK-positive if strong granular cytoplasmic brown staining present in the neoplastic cells[11]. Appropriate positive and negative controls were included in each experiment.

2.5. RNA and DNA isolation

Total RNA was isolated from frozen tissue according to a standard laboratory protocol using TRIzol (Life technologies, Carlsbad, USA). RNA from FFPE samples was isolated using the RNeasy FFPE kit according to the manufacturer's protocol (Qiagen, Venlo, The Netherlands). Genomic DNA from frozen tissue samples was isolated using a routine salt-chloroform protocol using standard protocols. The ReliaPrep™ FFPE gDNAMiniprep System kit (Promega, Madison, USA) was used to isolate DNA from FFPE samples following the protocol of the manufacturer. The NanoDrop (Thermo Fisher Scientific Inc., Waltham, USA) was used to determine DNA and RNA concentrations.

2.6. Transcriptome sequencing and fusion detection

Library preparation for paired-end RNA sequencing was performed using the TruSeq RNA kit (Illumina, San Diego, USA), starting from 500ng of total RNA. Paired-end reads of 100nt were generated on the Hiseq2500 (Illumina, San Diego, USA). We used two independent algorithms to predict presence of the fusion transcripts. DeFuse (v.0.6.1) and EricScript[12,13]. DeFuse maps the reads to the reference genome using an automated process which involves SAMtools, bowtie, BLAT and GMAP[14-17]. EricScript uses a series of alignment steps, by BWA and BLAT, to identify and precisely map discordant reads that point to gene fusions, after which the RNA-seq data are screened for the presence of spanning reads to support these putative fusions[18]. We excluded fusions derived either from read-through transcripts or

fusion genes that mapped to multiple genomic loci with high homology. We next focused on the fusion genes detected by both deFuse and EricScript. We inspected mapping of split reads and spanning reads using the University of California Santa Cruz (UCSC) genome browser. Predictions of the presence of an ORF in the fusion products were obtained from deFuse. RNA-seq data have been deposited on European Nucleotide Archive (ENA) website and are available under accession number: PRJEB12854.

2.7. Validation of the fusion products by RT-PCR

cDNA was synthesized with Superscript II reverse transcriptase and random primers according to the company instruction starting from 500ng total RNA (Invitrogen, Carlsbad, USA). PCR was performed using 10ng cDNA as input in a final volume of 30µl containing 1x PCR buffer and $MgCl_2$ (final concentration 1.5mM), 0.2µl Tag DNA polymerase (5unit/µl) (Invitrogen, Carlsbad, USA) and 500nM primers designed using Clone Manager Suite (Sci-Ed Software, Morrisville, USA) (Supplementary Table 6.1). Amplification consisted of 35 (frozen samples) or 45 (FFPE) cycles using a thermocycler (Bio-Rad, Hercules, USA). PCR products were analysed on a 3% agarose gel, purified using Zymoclean™ Gel DNA Recovery Kit (Zymo research, Irvine, USA) and sequenced at LGC Genomics (Berlin, Germany). Agarose gel pictures were captured using Gel Doc XR+ System (Bio-Rad, Hercules, USA).

2.8. Identification and validation of mutations in ALK, EGFR and KRAS gene

For each patient the RNA-seq bam file, generated by RSEM (1.2.9) was inspected in IGV[19]. All exons of *ALK*, *EGFR* and *KRAS* genes were visually screened for coverage and the presence of known resistance-associated mutations. To validate *ALK* mutations, 50ng of DNA was amplified as described above using primers designed with Clone Manager Suite (Sci-Ed Software, Morrisville, USA) (Supplementary Table 6.1). M13F or M13R tails were added to the 5' end of the primers designed for DNA to allow direct sequencing of the PCR products. Purification and sequencing was performed as described above. One of the *ALK* mutations was validated at the RNA level by RT-PCR and Sanger sequencing using a primer set that allowed specific amplification of the *EML4-ALK* breakpoint region.

2.9. Detection of ALK C1156Y and G1269A mutations by droplet digital PCR (ddPCR)

Mutant and wild type ddPCR primers and probes to detect C1156Y and G1269A ALK gene mutations were obtained from Bio-Rad (Hercules, USA). The ddPCR was performed on

18ng of genomic DNA as measured by Qubit (Life technologies, Carlsbad, USA) according to the manufacturer's instruction (Bio-Rad, Hercules, USA). Briefly, 11µl ddPCR Supermix for probes, 1µL of the mutation assay and genomic DNA were mixed in a final volume of 20µl. Droplets were generated using the QX100 Droplet generator after addition of 70µl droplet generation oil (Bio-Rad, Hercules, USA). PCR was performed on a T100 Thermal Cycler (Bio-Rad, Hercules, USA) using the following cycling conditions: 10 minutes at 95°C, 40 cycles of 95°C for 30 seconds, 55°C for 1 minute followed by 98°C for 10 minutes (ramp rate 2.5°C/sec). Samples were transferred to the QX200 Droplet Reader (Bio-Rad, Hercules, USA) for fluorescent measurement of FAM and HEX probes and data were analysed by Quantasoft software version 1.6.6 (Quantasoft, Prague, Czech Republic). In addition to the pre- and post-treatment tumour samples, 10 normal control samples were used as negative controls. Sensitivity of the assays was 0.1 and 0.5% for C1156Y and G1269A respectively, as determined on dilution series of the post-treatment tumour samples in combination with the total number of droplets that could be analysed in the primary tumour sample.

3. Results

3.1. Patients

The three patients, aged 27 to 56 years, were all tested positive for *ALK* IHC before crizotinib treatment (pre-treatment) and at disease progression (post-treatment). All three patients were *ALK* FISH positive both before crizotinib and at disease progression. Only patient #3 showed extra *ALK* copies in the diagnostic FISH analysis. In addition, *EML4-ALK* specific FISH revealed only one copy of this fusion per cell in this patient. Patient ALK3, ALK4 and ALK6 showed a partial response (PR) with progression free survival (PFS) of 6.8, 15.9 and 9.5 months, respectively (Table 6.1).

Patient ALK3 was diagnosed with adenocarcinoma in March 2011 and treated with two courses of cisplatin and pemetrexed in the same month. She received crizotinib from October 2011 and died in December 2012. Patient ALK4 was diagnosed with adenocarcinoma in May 2005 and received cisplatin and pemetrexed until June 2006, when a bilateral adnexectomy was performed for a large metastasis. In May 2008 she developed liver metastases and was treated with a single agent pemetrexed. She received crizotinib from November 2011 based on an *ALK*-positive FISH in the primary tumour sample and had a near complete response. In January 2013 she developed liver metastases, which were treated with metastasectomy and radiofrequency ablation. In July 2013, she started

Table 6.1. Patients' characteristics and fusion products detected in crizotinib resistant tumours

Patient	Biobank no.	Sample accession ID at ENA website	Age at diagnosis	Smoking	Tumour response (PFS in months)	Type	Tumour (%)	High confidence gene fusions	Predicted ORF
ALK 3	1211987	SAMEA3881068	27	None	PR (6.8)	Frozen	90	*EML4-ALK*	Yes
								NRG1-RBPMS	No
ALK 4	1305996	SAMEA3881070	34	None	PR (15.9)	Frozen	90	*EML4-ALK*	Yes
								CLIP4-VSNL1	Yes
								MCFD2-CLIP4	No
								KIAA0040-RFWD2	No
ALK 6	1219581	SAMEA3881069	55	Current	PR (9.5)	Frozen	70	*EML4-ALK*	Yes

PFS is progression free survival; ORF: Open reading frame.

treatment with ceritinib and had a complete response. Since then she is well and alive on maintenance ceritinib. Patient ALK6 was diagnosed with lung adenocarcinoma in March 2010 and received cisplatin and pemetrexed in December 2011. He received crizotinib from January 2012 and one year later switched to ceritinib. Treatment was ended in October 2013 and the patient died in January 2014.

3.2. Detection of fusion products

A total of 19.9, 28.9 and 19.9 million reads were aligned for post-treatment tumour samples of patient ALK3, ALK4 and ALK6, respectively. Seven fusion gene products were identified in these three tumour samples, including an *ALK* fusion gene in each patient (Table 6.1). The fusion partner was *EML4* in all three patients according to the deFuse and EricScript analysis. The breakpoint was in intron 20 of the *EML4* gene in patient ALK3 and intron 6 of the *EML4* gene in patients ALK4 and ALK6. The *EML4* gene was fused to exon 20 of the *ALK* gene in all three patients.

In patient ALK3 one additional fusion gene, i.e. *NRG1-RBPMS*, without a predicted ORF according to deFuse was detected. Patient ALK4 contained three additional fusion genes, one with and two without predicted ORFs (Table 6.1). Two of the fusion genes (*CLIP4-VSNL1* and *MCFD2-CLIP4*) were the result of multiple genomic aberrations at the *ALK* gene region on chromosome 2 (Figure 6.1A).

Both fusion products involved the *CLIP4* gene mapping 8kb downstream of the *ALK* gene. In one fusion transcript, exon 14 of the *CLIP4* gene was fused to exon 2 of the *VSNL1* gene, resulting in a fusion transcript with a predicted ORF. In the second fusion transcript, exon 15 of the *CLIP4* gene was fused to the non-coding exon 1 of the *MCFD2* gene. This fusion did not have a predicted ORF. In patient ALK6, no additional fusion products were identified.

3.3. Validation of the fusion products by RT-PCR

EML4-ALK fusion transcripts were confirmed by RT-PCR followed by Sanger sequencing in post-treatment tumour samples of patients ALK3, ALK4 and ALK6 (Figure 6.1B). We failed to validate the *NRG1-RBPMS* fusion in patient ALK3 on both the frozen and FFPE post-treatment sample, despite good amplification product for the house keeping gene (data not shown). This might be due to low expression level, or to design of a suboptimal primer sets, precluding efficient amplification. We next validated the three novel fusion products identified in patient ALK4. A PCR product of the expected size was observed for all three fusion genes in the frozen biopsy of the post-treatment tumour samples (Figure

6.1C). Sanger sequencing of these RT-PCR products confirmed the expected sequence consistent with the prediction of deFuse and EricScript. Next, we evaluated whether these fusion transcripts were also present in the pre-treatment FFPE tumour samples of these patients. FFPE samples of the post-treatment tumours were included as positive controls. The *CLIP4-VSNL1*, *MCFD2-CLIP4* and *KIAA0040-RFWD2* fusion transcripts were detected in the pre- and post-treatment tumour sample of patient ALK4 (Figure 6.1D).

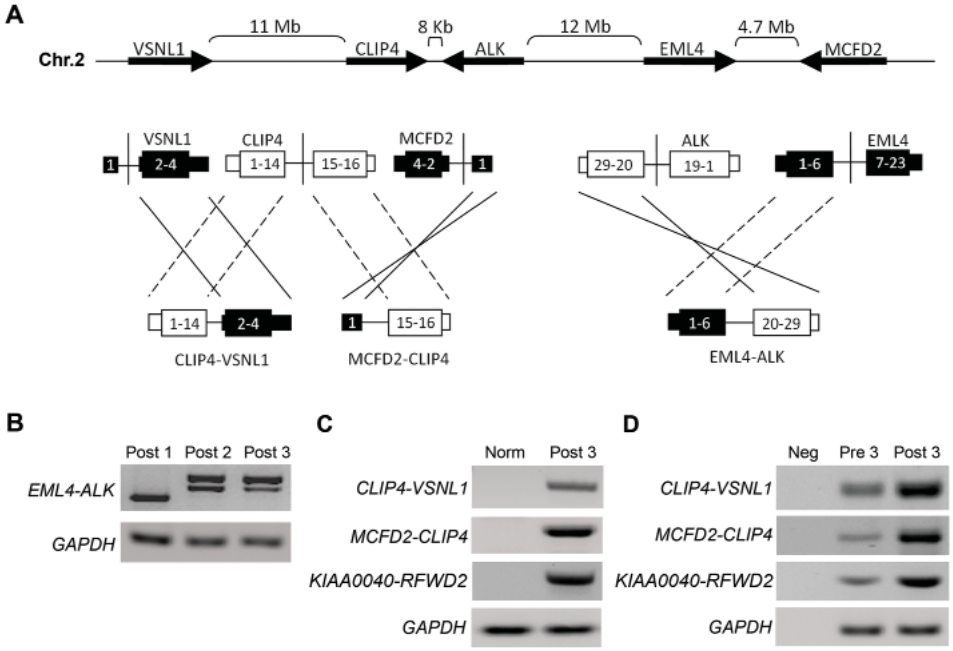

Figure 6.1. Schematic representation of fusion gene products clustered at the ALK locus and selected fusions validation.
(A) Three fusion products clustered at a 25Mb genomic region including the *ALK* gene locus in the tumour of patient ALK4. Two of the three fusion products are the result of an inversion (*EML4-ALK* and *MCFD2-CLIP4*), whereas the third fusion product is generated via an eversion (*CLIP4-VSNL1*). *EML4-ALK* and *CLIP4-VSNL1* contain a predicted ORF. **(B)** Detection of *EML4-ALK* fusion in three crizotinib post-treatment tumour samples (post 1, post 2 and post 3, corresponding to post-treatment samples of patient ALK3, ALK6 and ALK4 respectively). **(C)** Validation of three novel fusion genes in frozen post-treatment tumour sample of patient ALK4. **(D)** Detection of the fusion genes in FFPE samples of post-treatment sample of patient ALK4 and analysis of the fusion gene in pre-treatment tumour sample of patient ALK4. Norm: Normal lung tissue; Pre: pre-treatment tumour sample; Post: post-treatment tumour sample; Neg: Negative control.

3.4. Identification and validation of mutations in ALK, EGFR and KRAS

Mutations in *ALK*, *EGFR* and *KRAS* have been reported to confer resistance against crizotinib. To determine presence of mutations in these genes in the three post-treatment samples, we inspected the RNA-seq bam files in IGV. In patient ALK3 no *EGFR* gene mutations were observed, whereas for *KRAS* the coverage was too low. Analysis of the paired-end RNA-seq data revealed no mutations in the *EGFR* and *KRAS* genes in the post-treatment samples of patients ALK4 and ALK6 (Table 6.2). A mutation was found in 57% of the RNA-seq reads in the *ALK* gene, i.e. p.C1156Y (NM_004304.3:c.3467G>A), in patient ALK3. Sanger sequencing of the RT-PCR product using *EML4-ALK* fusion gene specific primers confirmed presence of both wild type and mutant *EML4-ALK* fusion gene transcripts consistent with the RNA-seq data (Figure 6.2A). In patient ALK4, an *ALK* mutation was observed in 100% of the RNA-seq reads, i.e. p.G1269A (NM_004304.3:c.3806G>C). Sanger sequencing confirmed presence of the mutations at the DNA level in the post-treatment tumours of both patients (Figure 6.2B). No mutations were observed in the *ALK* gene in the post-treatment sample of patient ALK6. No mutations were observed in the *KRAS* and *EGFR* genes (Table 6.2).

Analysis of the *ALK* mutations in the pre-treatment tumour samples of patients ALK3 and ALK4 by Sanger DNA sequencing revealed no mutations. To exclude presence of a minor clone with the *ALK* mutation in the pre-treatment tumour samples we performed ddPCR. In the pre-treatment samples no mutations were detected. In the post-treatment tumours, the fractional abundance of the corresponding mutant alleles was 26% and 19.8% in patients ALK3 and ALK4, respectively (Figure 6.2C).

Table 6.2. Summary of the diagnostic FISH, immunohistochemistry and the transcriptome analysis results

	Pre-treatment		Post-treatment					
Patient	ALK FISH (%)	ALK IHC	ALK FISH (%)	ALK IHC	ALK mutation	EML4-ALK duplication	EGFR mutation	KRAS mutation
ALK 3	>15	+	>50	+	p.C1156Y	+*	WT	Unknown
ALK 4	>15	+	>50	+	p.G1269A	none	WT	WT
ALK 6	>50	+	>50	+	WT	none	WT	WT

WT: Wild type; ALK-IHC is either positive or negative using D5F3 antibody for immunohistochemistry in combination with the Optiview system. *See discussion.

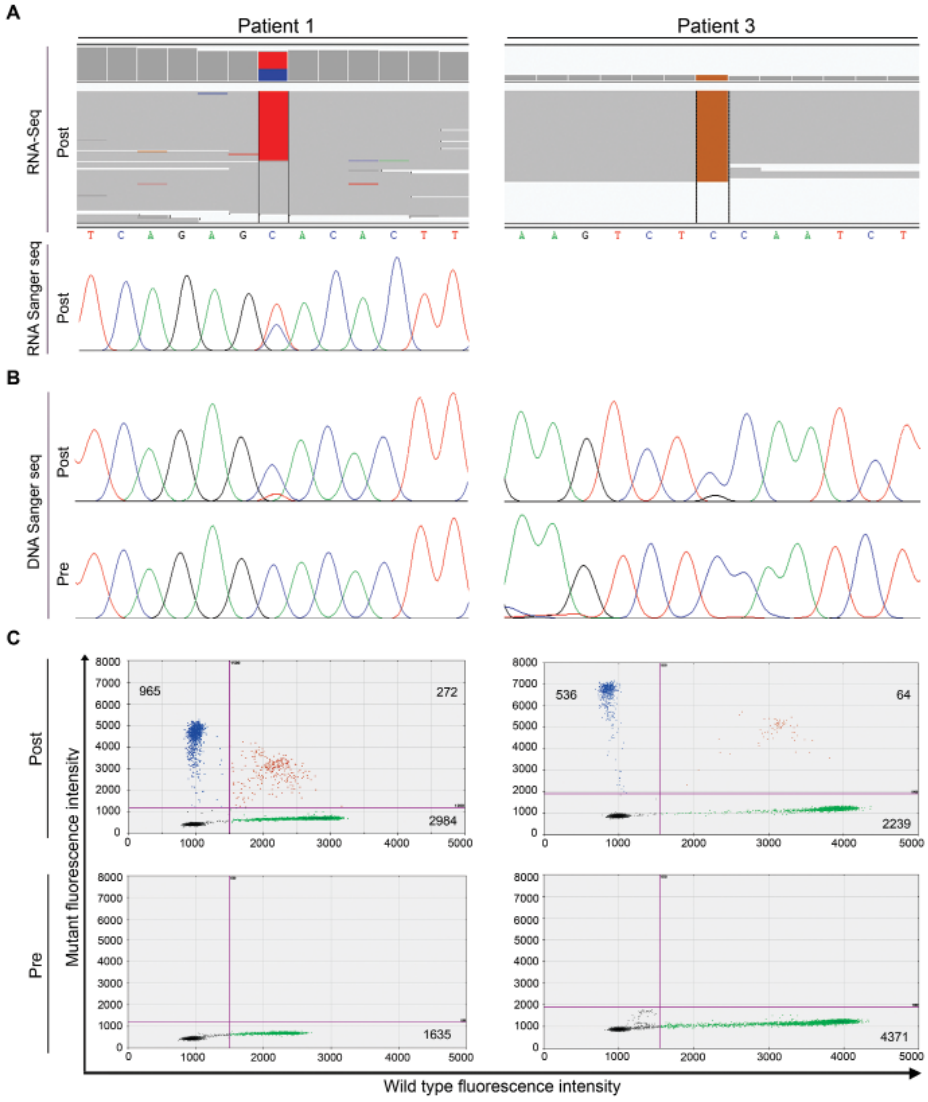

Figure 6.2. Detection of *ALK* gene mutations in tumour samples.
(A) RNA-sequencing reads of the two mutations. Grey bars show the wild type positions, the coloured bar indicates the mutant position. The number of wild type and mutant reads were 56/75 for patient ALK3 (c.3467G>A) and 0/25 for patient ALK4 (c.3806G>C) (Top). RNA Sanger sequencing in the post-treatment tumour sample of patient ALK3 confirmed presence of wild type and mutated *EML4-ALK* copy using primers covering the *ALK* break (Bottom). The sequences in this picture are based on plus strand, whereas the *ALK* gene is located on the minus strand of chromosome 2. **(B)** DNA Sanger sequencing results in the pre- and post-treatment tumour samples. **(C)** ddPCR results of the pre-and post-treatment tumour samples of patient ALK3 and ALK4. Number of positive droplets for mutant and wild type alleles is written in each gate of the scatter plots. Sensitivity of the assays was 0.1 and 0.5% for C1156Y and G1269A, respectively. Fractional abundance for the mutant allele was 26% and 19.8% in the post-treatment tumours of patients ALK3 and ALK4, respectively. Pre: pre-treatment tumour sample; Post: post-treatment tumour sample.

4. Discussion

ALK-break positive NSCLC patients respond to crizotinib in over 60% of cases, but after 9 to 12 months drug resistance develops in all patients[20]. In several studies mutations in the *ALK* gene or mutations in *KRAS* and *EGFR* in re-biopsies were observed as mechanisms of the resistance[1,9,21]. In this study we focused on detection of novel fusion products as possible resistance mechanisms to crizotinib using RNA-seq. In addition, we also evaluated the presence of hotspot mutations in *ALK*, *KRAS* and *EGFR* known to be associated with crizotinib resistance using the same RNA-seq data.

In patient ALK3, we confirmed the presence of the *EML4-ALK* fusion gene in the post-treatment sample taken from a tumour growing under crizotinib. One additional fusion gene without a predicted ORF was identified in this patient, but could not be confirmed by RT-PCR. A C1156Y *ALK* mutation was observed in approximately half of the RNA-seq reads in this patient. This suggests that the *ALK* mutation was gained after duplication of the *EML4-ALK* fusion or that the mutation is present only in a proportion of the tumour cells, while being wild type in the other tumour cells. The alternative explanation, i.e. gain of a de novo fusion gene in combination with gain of an *ALK* mutation on one of the two fusion genes seems unlikely. The mutation was not present in the pre-treatment biopsy using the sensitive ddPCR. In patient ALK4, we confirmed the presence of the *EML4-ALK* fusion gene in the post-treatment sample. Three additional fusion genes were detected, of which one had a predicted ORF. However, all three fusion genes were present in the pre-treatment sample, and thus not treatment induced. In addition, we observed a G1269A *ALK* mutation in the post-treatment tumour, which was not detectable in the pre-treatment tumour sample using ddPCR. Gain of an *ALK* mutation most likely caused the crizotinib resistance in patients ALK3 and ALK4. ALK-dependent crizotinib resistance mechanisms were thus involved in 2 of the 3 patients. Functional analysis of the two observed resistance-associated mutations in Ba/F3 and NIH3T3 cells has proven their role in crizotinib resistance[9,21].

The G1269A mutation is located close to the crizotinib binding site and induces a stronger resistance towards crizotinib than the C1156Y mutation[9].

The relative quick appearance of crizotinib resistance in patient ALK3 may be due to the combination of different post-treatment mechanisms, the milder C1156Y mutation and the potential *EML4-ALK* duplication. Moreover, based on the normalized RNA-seq reads, this patient had a 2 to 3 fold higher expression level of the *ALK* fusion gene as compared to the two other patients. Thus, despite gain of the less effective mutation, *EML4-ALK*

duplication and the higher expression level might also have contributed to the short PFS. A number of studies have investigated mechanisms of resistance to crizotinib in post-treatment tumour samples of NSCLC patients. *ALK* mutations were the most commonly observed aberrations identified in post-treatment biopsies of 16 out of 51 (31%) patients[1,9,21-24]. We detected *ALK* mutations in 2 of the 3 patients. Using ddPCR we showed that these mutations were not detectable in pre-treatment biopsies that is in agreement with the fact that these mutations are associated with resistance to crizotinib. *ALK* gain has been reported as resistance mechanism in 4 out of 36 (11%) patients[1,9,23]. We observed *EML4-ALK* RNA-seq reads with and without the *ALK* mutation in patient ALK3. This might indicate a mixed tumour cell population or duplication of the fusion gene with gain of an *ALK* mutation in one of the two copies of the *EML4-ALK* fusion gene. Of the 36 patients studied for both *ALK* mutations and *ALK* gain, only one case was positive for both.

In patient ALK6, we confirmed presence of the *EML4-ALK* fusion gene in the post-treatment sample. No additional fusion genes were identified. We did not find *ALK* mutations or gain of *ALK* copies, indicating the occurrence of an ALK-independent resistance mechanism. Also, we did not find evidence for the other currently known ALK-independent crizotinib resistance-associated aberrations in this patient. As the number of aligned reads in this patient was similar to patient ALK3 and we did detect the *EML4-ALK* fusion gene, it seems unlikely that we failed to detect other fusion genes. Moreover, we found no evidence of increased expression of *ALK* or *EGFR* in the RNA-seq data (results not shown). Other currently unknown ALK-independent resistance mechanisms might have been induced in this tumour sample.

In patient ALK4, three novel fusion gene products (one with and two without a predicted ORF) were present in both the pre- and post-treatment tumour samples. Given the gain of a functionally confirmed *ALK* mutation, it seems less likely that these fusions are associated with resistance to crizotinib. Moreover, these fusion products were already present in the pre-treatment tumour sample. The role of the three novel fusion gene products, one with and two without a predicted ORF in patient ALK4, remain unknown. The clustering of three fusion gene products within the *ALK* gene region suggests that this genomic region is an unstable region in advanced NSCLC. The frequent loss of (parts of) the short arm of chromosome 2 (2p14-16, 2p23.3 and 2p24.3) as observed in NSCLC is consistent with this region being susceptible to chromosomal breaks[25,26]. Based on the orientation of the genes, the FISH results and the two breakpoints in the *CLIP4* gene, it is most likely that the *CLIP4-VSNL1* and *MCFD2-CLIP4* are present on the same chromosome as a result of a

duplication followed by an inversion. The *ELM4-ALK* fusion gene might be present on the same or on the sister chromosome.

The question is what can be done for patients that become resistant to crizotinib. Besides, crizotinib and ceritinib that both show high tumour response rates, next generation ALK inhibitors such as alectinib, brigatinib (AP26113) and lorlatinib (PF-06463922) are under development and show high response rates in diverse resistance associated ALK mutants. For instance, ceritinib is active against crizotinib resistant ALK mutant forms such a L1196M, G1123S, G1269A, S1206Y and I1171T. Alectinib is active against L1196M, C1156Y, 1151T-ins, L1152R, F1174L, G1269A, and R1275Q. Brigatinib is active against L1196M, F1174L, G1269A, but not S1206Y. PF-6463922 is active against all the above-mentioned ALK mutant forms[27-30]. A recent study on a single patient with NSCLC has shown that crizotinib-resistant *ALK*-positive cells can be resensitised to crizotinib after treatment with lorlatinib via acquiring *ALK* L1198F mutation[31].

In conclusion, we identified four novel gene fusion products in two of three crizotinib resistant post-treatment tumour samples. In two patients gain of *ALK* mutations was the most likely resistance mechanisms. In the third patient, the putative ALK-independent resistance mechanism remained unclear. Overall, it is unlikely that the fusion genes identified in our study are involved in resistance to crizotinib.

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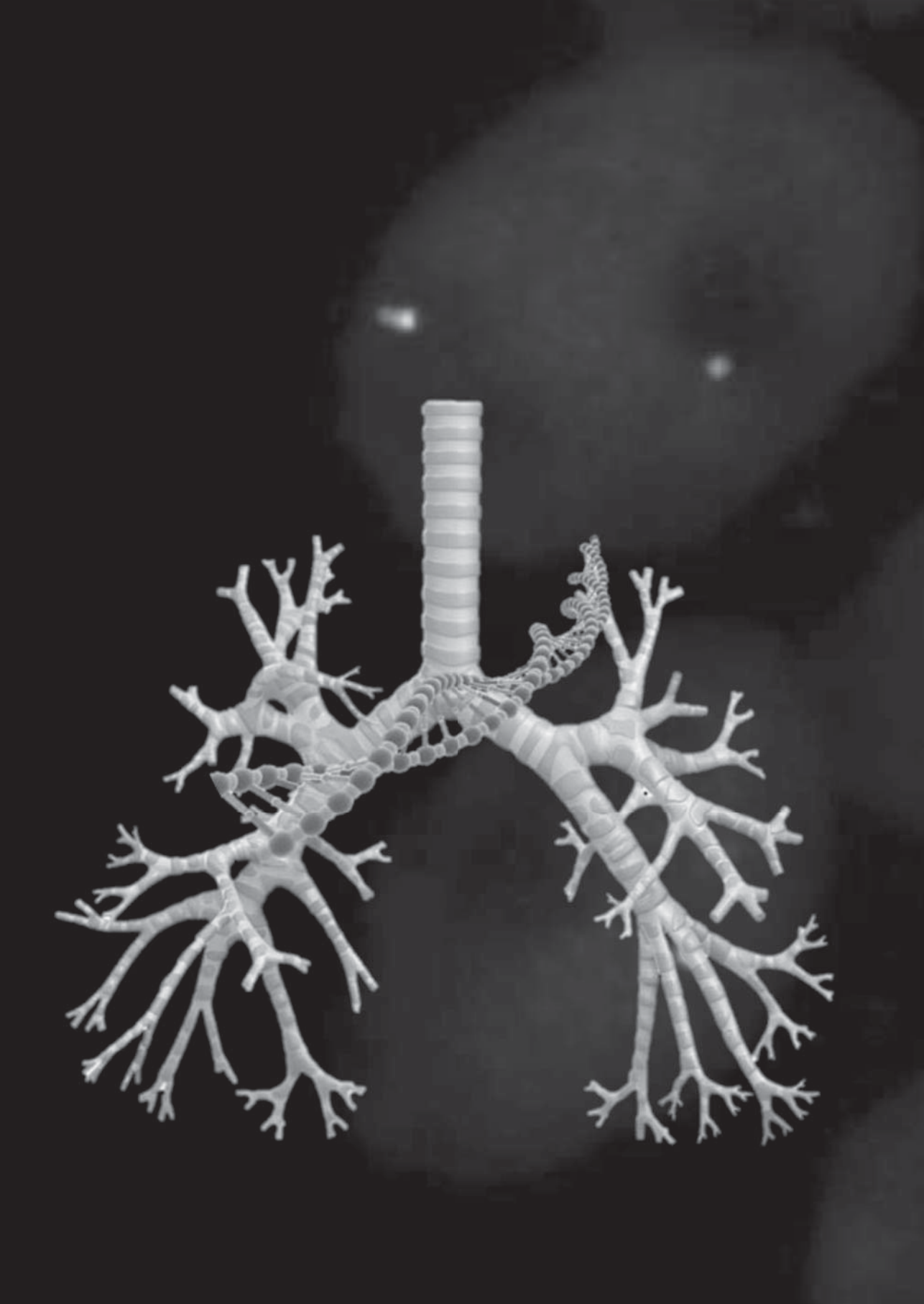
6. Supplementary information

Supplementary Table 6.1. List of primers for detection of fusion transcripts and ALK mutations in frozen and FFPE samples

Primer set	Name	Primer sequence	DNA/ RNA	Used in	Annealing (°C)	No. of cycles	Product size (bp)	Transcript Ensembl ID
1	EML4-E5-F ALK-E20-R	5′-ACGACCATCACCAGCTGAAA-3′ 5′-CTGATGGAGGAGGTCTTGCC-3′	RNA	Frozen	55	35	327/294	ENST00000318522 ENST00000389048
2	EML4-E20-F ALK-E20-R	5′-CAGATATGGAAGGTGCACTG-3′ 5′-CTGATGGAGGAGGTCTTGCC-3′	RNA	Frozen	55	35	252	ENST00000318522 ENST00000389048
3	EML4-E20-F ALK-E23-R	5′-GTCTAACTCGGGAGACTATG-3′ 5′-CCCCAATGCAGCGAACAATG-3′	RNA	Frozen	60	35	413	ENST00000318522 ENST00000389048
4	ALK-E22-F ALK-E22-R	5′-GTAAAACGACGGCCAGTTGGCTTGCGGACTCTGTAG-3′ 5′-GGAAACAGCTATGACCATGGGTGAGGGTGTCTCTCTGTG-3′	DNA	Frozen/FFPE	60	35	170	ENST00000389048 ENST00000389048
5	ALK-E25-F ALK-E25-R	5′-GTAAAACGACGGCCAGCTTCCCAGAGACATTGCTGC-3′ 5′-GGAAACAGCTATGACCATGCCATTCTTGAGGGGCTGAGG-3′	DNA	Frozen/FFPE	58	35	163	ENST00000389048 ENST00000389048
6	CLIP4-E13-F VSNL1-E3-R	5′-ATGGTTCAGTTGGAGGTGTG-3′ 5′-CTTGGAGGCGTCTCCATAAG-3′	RNA	Frozen	55	35	338	ENST00000320081 ENST00000404666

7	MCFD2 -E1-F CLIP4-E16-R1	5′-AGCCGAGGAAGAGCGTTTTG-3′ 5′-ATAGCGCTTGTCACCCACTG-3′	RNA	Frozen	55	35	380	ENST00000319466 ENST00000320081
8	KIAA0040-E3-F1 RFWD2-E13-R	5′-GAACGTGACCTCCAGGAAAG-3′ 5′-TGACCTCTGTCCTGTGAATC-3′	RNA	Frozen	55	40	356	ENST00000444639 ENST00000367669
9	CLIP4-E14-F VSNL1-E2-R	5′-GATTCCCTGGATACCCTTTC-3′ 5′-CCAGTTTGCTATTCTGCTTC-3′	RNA	FFPE	55	40	88	ENST00000320081 ENST00000404666
10	MCFD2 -E1-F CLIP4-E15-R2	5′-AGCCGAGGAAGAGCGTTTTG-3′ 5′-GGGAAGAAGCAGAAGTTGTG-3′	RNA	FFPE	55	45	123	ENST00000319466 ENST00000320081
11	KIAA0040-E3-F2 RFWD2-E12-R	5′-TGACAACGCAAAGCAAGAAG-3′ 5′-CAGCAATCGCAAAATAGTCAC-3′	RNA	FFPE	55	45	116	ENST00000444639 ENST00000367669

Underlined nucleotides: M13 primers.



7

Dichotomous ALK-IHC is a better predictor for ALK inhibition outcome than traditional ALK-FISH in advanced non-small cell lung cancer

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Abstract

Purpose

ALK rearrangement detection using fluorescence in situ hybridization (FISH) is the standard test to identify non-small cell lung carcinoma (NSCLC) patients eligible for treatment with ALK inhibitors. Recently ALK protein expression in resectable NSCLC showed predictive value. We evaluated tumour response rate and survival after crizotinib treatment of advanced NSCLC patients with ALK activation using both dichotomous immunohistochemical staining (IHC) and FISH.

Design

Stage IV NSCLC patients treated with crizotinib were selected. Tumour response was assessed. ALK rearrangements were detected by FISH (Vysis ALK-Break-Apart FISH-Probe KIT) and IHC (Ventana ALK-D5F3-CDx assay). Cohorts of ALK-FISH-positive advanced NSCLC patients from 4 other hospitals were used for validation.

Results

Twenty-nine consecutive patients with ALK-positive advanced NSCLC diagnosed by FISH and/or IHC on small biopsies or fine needle aspirations (FNA) were treated with ALK inhibitors. All ALK-IHC-positive patients responded to crizotinib except three with primary resistance. No tumour response was observed in 13 ALK-FISH-positive but ALK-IHC-negative patients. This was confirmed in an external cohort of 16 patients. ROC curves for ALK-IHC and ALK-FISH compared to treatment outcome, showed that dichotomous ALK-IHC outperforms ALK-FISH (tumour response AUC 0.86 vs. 0.64 p=0.03; PFS AUC 0.86 vs. 0.36 p=0.005; OS AUC 0.78 vs. 0.41 p=0.01, respectively).

Conclusions

Dichotomous ALK-IHC is superior to ALK-FISH on small biopsies and FNA to predict tumour response and survival to crizotinib for advanced NSCLC patients. Our data strongly suggest adapting the guidelines and using dichotomous ALK-IHC as standard companion diagnostic test to select NSCLC patients that benefit from ALK-targeting therapy.

1. Introduction

In 2007, the first report of an Echinoderm microtubule associated protein like 4 - Anaplastic Lymphoma Kinase (*EML4-ALK*) fusion gene on chromosome 2p in lung cancer has been published[1]. *EML4-ALK* variants with different breakpoints and other fusion partners for *ALK* have been identified, e.g. *KIF5B*, *TRK*, *TPR*, *KLC1*, *DCTN* and *SQSTM*[1-4]. *EML4-ALK* rearrangement occurs in approximately 5% of advanced non-squamous non-small cell lung carcinoma (NSCLC) patients[1,5-7]. In stages I to III NSCLC ALK rearrangement frequency is 3.2%[8].

In 2010, crizotinib has been registered as the first drug for treating EML4-ALK positive patients with advanced NSCLC. Phase II studies in patients with ALK-break positive NSCLC defined by FISH revealed tumour response rates up to 65%[4,9]. Phase III studies confirmed the response rate and showed an improved survival compared to chemotherapy[10]. In these studies no major differences were observed between ALK-rearrangements at DNA, expression of fusions at RNA, and ALK detection at protein level. However, the explanation why some patients with an ALK-rearrangement did not respond to initial ALK inhibitors was not solved.

In the US the CE-IVD-marked Abbott FISH kit was registered as companion diagnostic for crizotinib. The Ventana ALK (D5F3) CDx assay is approved as a CE-IVD in Europe, China (www.ventana.com) and US (www.fda.gov) since 2012, 2013 and June 2015, respectively. This test is used on a fully-automatic BenchMark® XT and with a dichotomous scoring system. This test is unambiguous and only provides a positive or negative outcome[11]. In Japan, ALK fusion gene detection by RT-PCR is most commonly used in the diagnostics of ALK-status[12]. In Europe, the European Medicines Agency (EMA) allows the use of clinically validated methods to detect ALK positivity either by FISH or IHC. The FDA-approved Abbott Break-Apart ALK FISH probe and scoring using the international guidelines (www.Abbott.com) is labour-intensive, time consuming and operator dependent[13]. Furthermore, the detection of ALK rearrangements by FISH is not always concordant with ALK protein expression, although most studies show a concordance close to 100%[7,8,12,14,15]. Recently, several papers have been published on the comparison of different ALK-IHC and ALK-FISH tests. Most of these studies used resection samples of which tissue microarrays (TMA) were made[8,12,14,15]. In those studies only few patients have been treated with crizotinib, probably at recurrence of disease. However, in clinical practice most ALK positive NSCLC patients present with stage IV disease upon diagnosis. This is often based on small

biopsies and/or fine needle aspiration (FNA), which is usually not enough to build TMAs. In previous studies, tests have been compared without investigating the predictive value for treatment outcome.

Therefore, we explored the predictive value of Ventana ALK (D5F3) CDx assay and the Abbott Break-Apart ALK FISH test, on small biopsies and FNA, taken for predictive screening in our referral centre. Cases were those routinely tested and those where the interpretation of the FISH test result was difficult. All patients were included for tumour response to crizotinib in stage IV NSCLC patients. We hypothesized that dichotomous ALK-IHC, measuring the expression of the ALK protein containing the active kinase part, has a better predictive value than ALK-FISH for ALK positive patients with high tumour responses and prolonged survival[11].

2. Methods

2.1. Patients

2.1.1. Screening group

All consecutive patients with stage IV non-squamous carcinoma tested in our national referral centre for ALK-break detection by FISH, or by dichotomous ALK-IHC scoring algorithm resulted in a selected cohort of cases who had a positive ALK test either by ALK-FISH. Consecutive patients were those with routinely established FISH and a highly selected group with difficult interpretable FISH test results. From 2013 in parallel to ALK-FISH, analysis was performed with Ventana IHC-ALK. Tissue blocks used for ALK-FISH were collected from all patients with ALK-FISH-positive tumours, but without ALK-IHC data (mainly before 2013). When sufficient neoplastic cells were present dichotomous ALK-IHC was performed. Twenty-nine ALK positive patients (either FISH or IHC positive) were treated with ALK inhibitors and prospectively evaluated for patients' characteristics, e.g. smoking and performance score, and treatment outcome. Tumour response was assessed on CT using RECIST 1.1[16].

2.1.2. External validation group

An external validation set of ALK-FISH positive tumour samples from patients treated with crizotinib in 4 other hospitals were collected. To exclude laboratory quality differences (e.g. FISH operator dependency etc.) between hospitals as potential bias, exchange of tumour

samples and test results is necessary. Samples were tested with the dichotomous ALK-IHC at UMCG. Response rates, duration of crizotinib treatment and survival ALK-FISH results were compared to dichotomous ALK-IHC results.

2.2. Detection of ALK status by Fluorescence in situ hybridization

FISH was performed with the commercial LSI ALK dual colour, break-apart rearrangement Probe KIT (Abbott Molecular Inc. 06N38-020, Des Plaines, IL) for the evaluation of ALK genomic status (ALK-FISH). In brief, 4µm FFPE tissue or CytoLyt® block sections were mounted on positively charged glass slides and dried overnight at 60°C. Areas with sufficient neoplastic cells were marked after review of the corresponding haematoxylin and eosin (H&E) slide by a pathologist. After deparaffinization, slides were pre-treated in TRIS/EDTA pH9.0 buffer at 120°C for 7 min in a pressure cooker, washed and incubated in RNase (0.1 mg/ml diluted in 2*SSC) at 37°C for 1h, washed again and treated with pepsin (0.1% diluted in 0.01 N HCL) at 37°C for 10 min (biopsies and fine needle aspiration 5 min), denaturation (12 min 80°C) and hybridization (overnight 37°C) were performed. After hybridization slides were washed 2*SSC/0.3%NP-40 73°C for 2 min followed by 2*SSC/0.1% NP-40 1 min at room temperature, dehydration, air-dried (2x alcohol 96% and air-dried) and finally, slides were mounted manually in vectashield with 0.33 µg/ml DAPI. Stained sections were stored at 2-8°C in the dark until evaluation was performed to prevent fading of the fluorescent signals. Using the appropriated filters, scoring was performed according to the international guidelines (www.Abbott.com) and analysed independently by two experienced FISH evaluators[13]. A case was considered ALK-FISH positive when >15% of the evaluated 100 neoplastic nuclei had a break-apart pattern. In case of discordance a third independent experienced assessor scored the sample. FISH was performed in the laboratory of Molecular Pathology at the University Medical Centre Groningen (Groningen, Netherlands). Moreover, in case enough tumour tissue was available, ALK-FISH was also performed at an international ALK-FISH laboratory (Prof. dr. P. Pauwels, Antwerp, Belgium), to confirm our test data. The ALK-FISH for the external validation cohort was performed in the local laboratories of MUMC+, ZGT, VUMC and RadboudUMC.

2.3. Detection of ALK expression status by immunohistochemistry and dichotomous scoring

ALK-IHC was performed on 4 µm-thick formalin fixed, paraffin-embedded tissue sections or CytoLyt® block sections using the Ventana ALK (D5F3) CDx assay on a Ventana BenchMark® XT automated slide-processing system (Ventana Medical Systems Inc., Tucson, AZ). Briefly,

slides of NSCLC tumour were subjected to deparaffinization using EZ Prep (VMSI) and 'extended' Cell Conditioning 1. Tissue sections were incubated with anti-ALK antibody (clone D5F3, VMSI) for 20 min. OptiView DAB IHC Detection Kit (VMSI) and OptiView Amplification Kit (VMSI) were used according to the manufacturer's recommendations for the visualization of the bound primary antibody (http://www.uclad.com/newsletters/ALK-LUNG-IHC-INTERPRETATION-GUIDE.pdf)[11]. Tissue slides were counterstained with Haematoxylin II and Bluing Reagent (VMSI). An ALK-positive cell line embedded in agar/FFPE or tissue sections of normal appendix containing ALK-positive ganglion cells were used as dichotomous ALK-IHC external controls in each run (VMSI). For evaluating the staining results, a dichotomous scoring system (positive or negative for ALK status) was used (package insert for Ventana anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody, Cat. No. 790-4794 / 06679072001). ALK expression was assessed independently by one trained scientist and one pathologist without knowledge of the FISH results or treatment outcome. Strong granular cytoplasmic staining of neoplastic cells (any percentage) was considered positive, only[11].

2.4. Validation of the fusion products by RT-PCR

cDNA was synthesized using 500ng total RNA input with Superscript II reverse transcriptase and random primers according to the company instruction (Invitrogen, Carlsbad, CA). PCR was performed using 10ng cDNA as input in a final volume of 30µl containing 1x PCR buffer and $MgCl_2$ (final concentration 1.5 mM), 0.2µl Tag DNA polymerase (5unit/µl) (Invitrogen, Carlsbad, CA) and 10mM primers designed using Clone Manager Suite (Sci-Ed Software, Morrisville, NC). Amplification consisted of 45 cycles using a thermocycler (Bio-Rad, Hercules, CA). Primers detecting the known *EML4-ALK* fusion gene variants are listed in Supplementary Table 7.1.

2.5. Statistical analysis

Statistics for patient characteristics were descriptive and Chi-square test was used for comparison. ALK test performance of FISH and the dichotomous IHC for screening and validation were compared with receiver operating characteristics (ROC) method with primary outcome tumour response and survival. To test for confounders, uni- and multivariate analyses were performed. Progression free survival (PFS) was defined from the date of treatment start until the date of disease progression, or death. Overall survival (OS) was defined from treatment start until the date of death. To compare survival of different

groups by Kaplan-Meijer method, log-rank test was used. For evaluating the influence of the percentage of breaks by FISH on PFS and OS, t-test was used. P values less than 0.05 were considered significant. Statistical analyses were performed with SPSS 22.0 (Armonk, NY).

3. Results

Between January 2011 and July 2015, 29 patients with advanced non-squamous NSCLC showed a positive ALK-status (Supplementary Figure 7.1), based on either ALK-FISH (n=16), ALK-IHC (n=3), or both (n=10). They were selected at a University Medical Centre and treated with crizotinib with a median follow up of 5·8 months (95% CI, 0.0-15.4). In 22/30 patients ALK tests were performed on endobronchial biopsies or transthoracic punctures and in 8 patients on FNA obtained by endoscopic ultrasound (EUS) and/or endobronchial ultrasound (EBUS). The median age of the screening cohort was 58 years (range: 21-79). Twenty patients were diagnosed with adenocarcinoma and ten with NSCLC favouring adenocarcinoma. Most patients were non-smokers (9/29) or previous smokers (11/29), and had a good performance score of 0-1 (Table 7.1). Crizotinib was used as 2nd line treatment after chemotherapy in 22/29 patients, others as 1st line therapy.

Table 7.1. Patient characteristics of screening cohort of ALK FISH positive patients with advanced non-squamous NSCLC grouped based on dichotomous ALK IHC result

	ALK IHC +	ALK IHC -	p value
No of patients	13	13	
Male/Female	2/11	8/5	0.02
Median age (range)	55 (21-73)	61 (38-79)	0.41
ECOG performance score			
0 - 1	11	12	0.78
2	1	0	
3	1	1	
Smoking status			
Never smoker	6	3	0.35
Current smoker	2	5	
Former smoker	5	5	
Time on crizotinib months (95% CI)	8.4 (3.1-13.7)	1.5 (0.9-2.1)	<0.001

In 26/29 patients dichotomous ALK-IHC could be performed.
Available tissue blocks previously used for ALK-FISH do not contain any neoplastic cells and dichotomous ALK-IHC and could therefore not be performed in 3 patients.

3.1. Treatment response of ALK-positive NSCLC patients as determined by Break-Apart FISH analysis

Twenty-six out of 29 patients were ALK-FISH positive with >15 of 100 neoplastic nuclei showing a break-apart pattern. Response rate (RR) in the FISH positive group, including thirteen dichotomous ALK-IHC negative patients, was low (23%), the median progression free survival (mPFS) was 1.8 months (95% CI, 1.4-2.2) and median overall survival (mOS) of 5.3 months (95% CI, 0.0-15.4). Three patients who were dichotomous ALK-IHC positive and FISH negative had a RR of 67% with a mPFS of 5.0 months (95% CI, 0.0-11.2) and mOS of 5.8 months (95% CI, 0.5-11.1).

The median percentage of neoplastic nuclei with ALK breaks was 35% (range: 2-78%). No association was observed between the percentage of ALK breaks and treatment outcome as determined by RR, PFS and OS (Table 7.2). Also, cases with an ALK-rearrangement based on a loss of the 5'-ALK-FISH-probe resulting in a pattern with a single red FISH signal were found in 6/30 patients. This FISH pattern was not associated with a better treatment outcome. The international ALK-FISH referral centre confirmed our ALK-FISH data in 14 cases of which enough tumour tissue was available.

3.2. Treatment response of dichotomous ALK-IHC positive NSCLC patients

In 26 out of 29 patients, tumour tissue with sufficient neoplastic cells was left to perform the dichotomous ALK-IHC test. Thirteen tumours showed ALK expression, whereas 13 were negative (all 13 were ALK-FISH positive). In the dichotomous ALK-IHC positive group the RR was 69% with a mPFS of 8.4 months (95% CI, 3.1-13.7) and a mOS of 18.3 months (95% CI, 12.4-24.2). No responses were observed in the dichotomous ALK-IHC negative group with a mPFS of 1.5 months (95% CI, 0.9-2.1) and a mOS of 5.0 months (95% CI, 4.3-5.7). Univariant and multivariate analyses revealed that dichotomous ALK-IHC was associated with better treatment outcome as determined by tumour response ($p<0.001$), mPFS ($p<0.001$) and mOS ($p=0.01$). The duration of tumour responses is outlined in Figure 7.1. Three patients with a dichotomous ALK-IHC positive tumour had no response to crizotinib, one patient died due to complications of brain radiotherapy shortly after starting crizotinib (ALK24). Another patient died due to liver failure due to metastases with necrosis on therapy (ALK20). In another patient only a part of the tumour at the border of the tissue section showed ALK-FISH positivity, while most neoplastic cells were ALK-FISH negative. Retesting of the residual tissue using both ALK-FISH and dichotomous ALK-IHC revealed that the earlier observed FISH-positive tumour area was not present anymore. All neoplastic

Table 7.2. Overview of the ALK test results in the screening cohort and clinical outcome in 29 stage IV NSCLC patients treated with crizotinib

Patient	ALK IHC	FISH	Copies	RT-PCR	Response	PFS (mo)	OS (mo)	Variants in EML4/ ALK-RT-PCR
ALK 1	+	45	N/A	+	PR	12.0	18.3	var 1
ALK 2	N/A	15	N/A	-	PD	1.4	13.9	
ALK 3	+	15	ER	+*	PR	6.8	13.8	E20;A20-var 2
ALK 4	+	2	1	+	PR	15.9	47.5	E6;A20-var 3
ALK 5	+	6	1-4	N/A	PR	5.0	5.8	
ALK 6	+	78	1-2; ER	+	PR	9.5	24.5	E6;A20-var 3
ALK 7	+	69	1-3	N/A	PR	39.0	39.0	
ALK 8	N/A	52	ER	-	PD	1.6	2.4	
ALK 9	-	25&	1-2	-	PD	0.2	0.9	
ALK 10	+	55	1-2	N/A	SD	9.6	14.2	
ALK 11	+	59	N/A	N/A	PR	22.0	31.0	
ALK 12	-	22	N/A	N/A	PD	3.5	10.1	
ALK 13	-	35	2-4	N/A	PD	1.9	16.1	
ALK 14	+	47	1-2	+	PR	8.4	31.5	E6;A20-var 3
ALK 15	-	15	N/A	N/A	PD	1.0	2.1	
ALK 16	+	64	ER	+	PR	4.1	18.9	E6;A20-var 3
ALK 17	N/A	20	1	N/A	PD	1.5	3.1	
ALK 18	-	35	ER	N/A	PD	0.5	0.5	
ALK 19	-	64	ER	N/A	PD	2.6	11.2	
ALK 20	+	47	1	N/A	PD	1.6	1.9	
ALK 21	+$	12	1	N/A	PD	1.1	2.5	
ALK 22	-	21	1	N/A	PD	2.0	5.0	
ALK 23	-	19	1	N/A	PD	1.3	5.3	
ALK 24	+	40	1	N/A	PD	0.9	0.9	
ALK 25	-	17	1	N/A	PD	1.5	4.6	
ALK 26	-	18	1	N/A	PD	0.7	4.4	
ALK 27	-	66	1	N/A	PD	0.7	4.8	
ALK 28	-	41	1	N/A	PD	1.8	5.2	
ALK 29	-	16	1	N/A	PD	1.1	6.0	

Dichotomous ALK-IHC is called either positive or negative and N/A means no neoplastic cells in the available pre-treatment biopsy; ALK-FISH is positive if >15% of 100 counted neoplastic nuclei show the defined break-apart patterns; *: ALK translocation confirmed by RNA seq (Saber et al.[30]); &: ALK-FISH positive (ALK-IHC not tested in 2012) in very small area of tumour tissue (<2%), the largest part of tumour is ALK-FISH, ALK-IHC and EML4-ALK-RTPCR negative; $: dichotomous ALK-IHC shows heterogeneous immunostaining of positive and negative neoplastic cells (see Figure 7.4B), PR= partial response, SD= stable disease, PD= progressive disease, ER= extra red (5' allele).

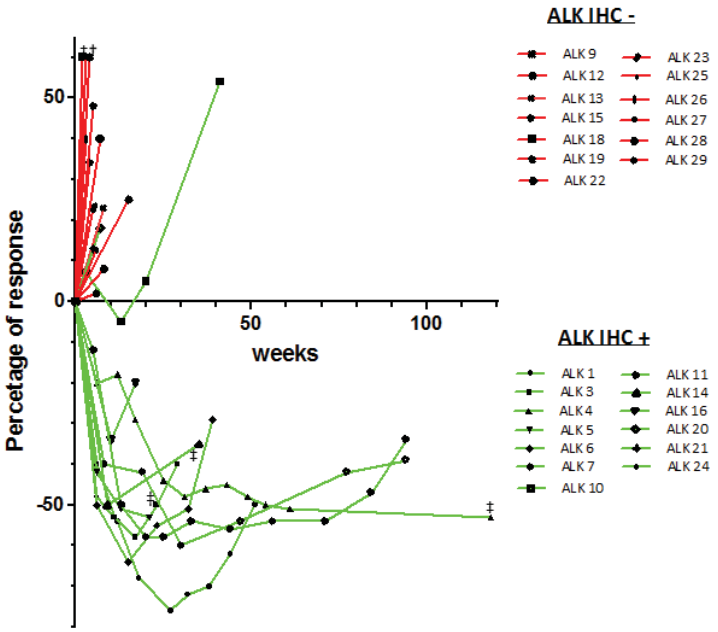

Figure 7.1. Tumour response duration upon crizotinib in 26 patients with ALK-FISH positive advanced non-squamous NSCLC stratified in dichotomous ALK-IHC positive and negative tumours from the screening cohort. Dichotomous ALK-IHC is depicted as positive (green) or negative (red) for tumour response outcome, measured on CT using RECIST v1.1. Tumour progression is determined when there is >20% increase in size, partial response is determined when there is >30% decrease in size, stable disease is determined between 20% increase and 30% decrease in size. Time of tumour response is in weeks. †: deceased, ‡: progressive disease due to new lesion.

cells were negative for both ALK-FISH and dichotomous ALK-IHC (ALK09; Figure 7.2A). This could explain lack of tumour response. In the third resistant patient (ALK21; Figure 7.2B) dichotomous ALK-IHC showed heterogeneous immunostaining of the neoplastic cells. Since all other dichotomous ALK-IHC positive cases in this study showed a homogeneous strong ALK-expression of all neoplastic cells (see Fig 2C and 2D for examples), the presence of the ALK-negative neoplastic cells in case ALK21 might explain the short PFS. One patient (ALK 10) without a tumour response had stable disease for 10 months.

3.3. Comparison of Break-Apart FISH, EML4/ALK-RTPCR and dichotomous ALK-IHC

Comparison of both, dichotomous ALK-IHC and ALK-FISH test performances showed a better prediction of tumour response shifting the ROC from 0.64 to 0.86 (p=0.03) in favour of dichotomous ALK-IHC (Figure 7.3). This has been observed for survival outcomes as well.

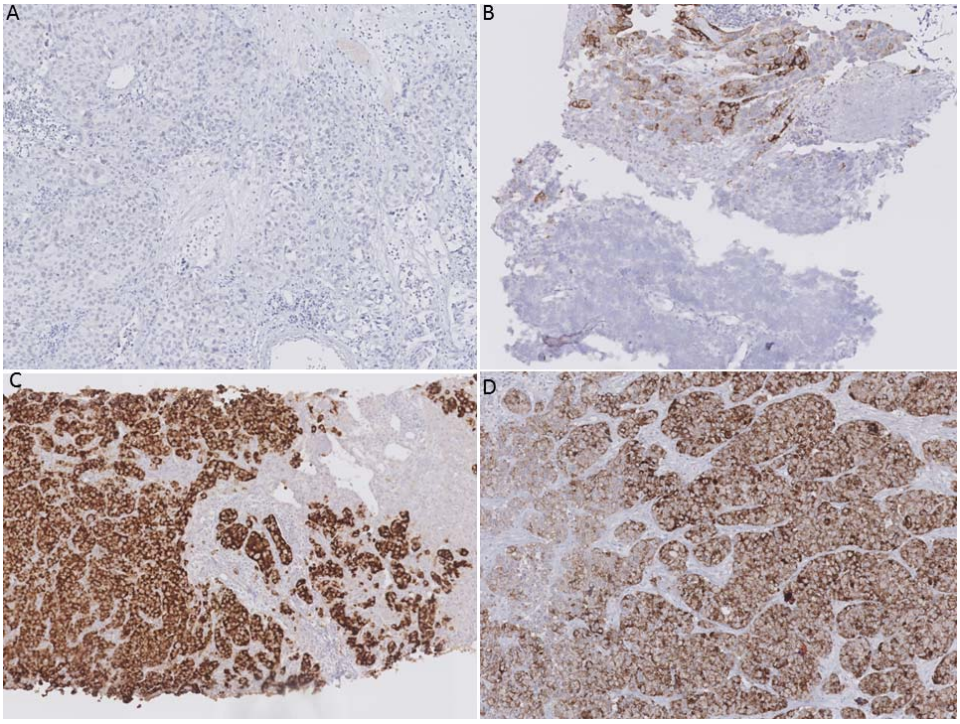

Figure 7.2. Normal and exceptional staining patterns in dichotomous ALK-IHC positive patients using Ventana ALK (D5F3) CDx assay.
A: ALK09 shows no immunostaining of the neoplastic cells (ALK-IHC-negative). B: ALK21 shows heterogeneous immunostaining of the neoplastic cells. C and D: Examples of the common homogeneous strong ALK-expression of all neoplastic cells (ALK-IHC positive).

Using PFS as the primary outcome, ROC increased from 0.36 to 0.86 (p=0.005) and for OS ROC increased from 0.41 to 0.78 (p=0.01). When dichotomous ALK-IHC and ALK-FISH data were combined, tumour response and survival was associated with ALK-IHC, independent of ALK-FISH outcome in multivariate analysis. Dichotomous ALK-IHC outcome was not associated with the percentage of neoplastic nuclei with ALK break apart patterns (range: 2-78%; Supplementary Figure 7.2), which is in line with a previous study[17]. To explain the discrepancy between FISH and IHC we performed RT-PCR or exome sequencing. In one patient (ALK04), RT-PCR confirmed the presence of ALK rearrangement (E20;A20, variant 2) that was not detected by ALK-FISH. This patient had one of the longest PFS in our treatment cohort (15.9 months). Patient ALK09 was positive for ALK-FISH in a small area of neoplastic cells only (<2%). Dichotomous ALK-IHC and EML4/ALK-RT-PCR were both negative on the

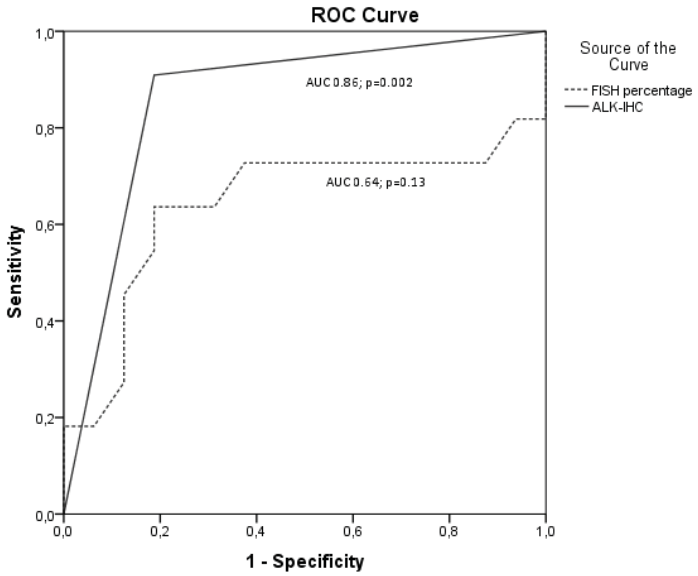

Figure 7.3. Receiver operating characteristic plot of dichotomous ALK-IHC Ventana and the Abbott break apart ALK-FISH to predict tumour response to crizotinib.
. In our cohort there were ALK-FISH positive patients without a tumour response to crizotinib, while all except 2 patients with dichotomous ALK-IHC responded. The ROC curves with survival as endpoint were similar. P-value relates to comparison with area under the ROC curve of 0.5.

same tissue block that did not contain the earlier small FISH-positive area anymore. This patient did not respond to crizotinib. In two patients, dichotomous ALK-IHC could not be performed, ALK-FISH was positive and EML4/ALK-RT-PCR was negative. Both patients did not respond to crizotinib. In five responding patients all three tests (dichotomous ALK-IHC, ALK-FISH, and ALK-RT-PCR) were positive (Table 7.2).

3.4. Validation cohort of dichotomous ALK-IHC compared to response

In the four Dutch hospitals 53 patients were treated with crizotinib on the basis of a positive ALK-FISH (Supplementary Figure 7.1). Of those, 16 patients had enough tumour tissue available to perform the dichotomous Ventana (D5F3) ALK-IHC. Eleven patients had a positive dichotomous ALK-IHC and 5 were negative. All 5 dichotomous ALK-IHC negative patients did not have a response to crizotinib. Response rate in the IHC positive patients was 72%. Three positive patients showed stable disease and 8/11 patients had a partial or complete response with crizotinib (Table 7.3).

Table 7.3. Overview of the validation cohort with ALK immunohistochemistry, FISH and tumour responses to crizotinib in 16 stage IV NSCLC patients from 4 hospitals treated with crizotinib

Patient	ALK IHC	FISH	Response	PFS (mo)	OS (mo)
EV1	-	+	PD	3.9	3.9
EV2	+	+	PR	10.3	45.6
EV3	+	+	PR	4.4	9.2
EV4	+	+	CR	19.7	20.1
EV5	+	+	SD	4.9	8.2
EV6	+	+	PR	10.6	17.5
EV7	+	+	PR	6.1	9.2
EV8	+	+	SD	1.9	5.1
EV9	+	+	PR	1.4	1.4
EV10	-	+	PD	1.8	3.7
EV11	+	+	SD	10.6	10.6
EV12	+	+	PR	13.8	15.6
EV13	-	+	PD	4.0	7.6
EV14	+	+	PR	6.2	8.7
EV15	-	+	PD	1.0	1.7
EV16	-	+	PD	1.6	18.1

Dichotomous ALK-IHC is called either positive or negative; ALK-FISH is positive if >15% of 100 counted neoplastic nuclei show the defined break-apart pattern; CR= complete response PR= partial response, SD= stable disease, PD= progressive disease.

4. Discussion

This is the first systematic study in advanced NSCLC with small amounts of tumour tissue to compare the value of the dichotomous ALK-IHC and ALK-FISH test on the basis of tumour response and survival as primary outcome. The dichotomous ALK-IHC largely outperformed the ALK-FISH as measured by ROC in a screening (one hospital) and validation (4 other hospitals) cohort of advanced ALK positive NSCLC patients. None of the patients with a negative dichotomous ALK-IHC responded to crizotinib. Therefore, patients who are tested with the Ventana ALK (D5F3) IHC do not need additional testing by ALK-FISH. This is in contrast to current guidelines (e.g. CAP/IASL/AMP 2013) that ALK-FISH is required to confirm ALK-IHC status. We demonstrated that these guidelines would be harmful for

ALK-IHC-positive/ALK-FISH-negative patients, who would not be eligible for treatment with appropriate ALK inhibitors.

Despite the large number of reports on the prevalence of ALK-positivity in NSCLC and the comparison of various detection assays, few studies report on the optimal biomarker predicted by tumour response to ALK inhibitors. ALK-IHC with 5A4 antibodies were used in comparison with Vysis FISH in a French study of 45 patients, an Italian study with the ETOP consortium and a Japanese study[7,12,14]. Importantly, none of these studies took patient outcome as a measurement of test efficacy. Other ALK-IHC and ALK-FISH tests are described in supplementary file.

The low response rate for the whole group was due to a substantial number of patients with ALK-FISH positive and ALK-IHC negative patients, indicating a selected population. None of those patients did respond to crizotinib. The higher frequency of such patients was due to the fact that our centre not only performed routine FISH testing, but also included cases with difficult interpretable FISH testing results. Therefore we asked an international ALK-FISH centre to confirm our ALK-FISH results. The dichotomous ALK-IHC positive group showed response rates and survival comparable to second line treatment as shown in the studies by Kwak et al. and Shaw et al.[4,10]. Moreover, two dichotomous ALK-IHC positive, but FISH-ALK negative patients responded to crizotinib. This is similar as in a study by Pekar et al., where two such patients also responded to crizotinib. These (ALK-IHC positive and FISH-ALK negative) patients showed an ALK translocation as measured with next generation sequencing (NGS)[18]. Different other patient reports showed a similar outcome[14,19,20]. Comparison of ALK expression levels with the presence of ALK rearrangements detected with FISH revealed an agreement between 90 and 100%[11,21-28].

Occasionally patients have been described that were ALK-FISH-positive, but ALK-IHC-negative and who responded to crizotinib. Ilie et al. showed three patients (EML4/ALK-break RT-PCR negative also) that responded due to the fact that they were cMET amplification positive, as crizotinib is a MET inhibitor as well[29]. This could also be the case for the patient mentioned in the paper by Marchetti et al.[8], although this was not evaluated. We also showed that heterogeneous staining was only observed in patients who did not respond to treatment. So, only those patients who have a homogenously positive dichotomous ALK-IHC staining respond effectively to ALK treatment.

In conclusion, this is the largest report where dichotomous ALK-IHC and FISH tests are evaluated in small biopsies and cytology alone. Dichotomous ALK-IHC (either positive or negative) outcome is superior to ALK-FISH on small biopsies and FNA to predict tumour

response and survival to anti-ALK therapy for advanced NSCLC patients. Our data strongly suggest that guidelines should be adapted and dichotomous ALK-IHC should be the standard companion diagnostic test to select NSCLC patients that benefit from anti-ALK therapy.

5. References

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6. Supplementary data

6.1. Different ALK-FISH and ALK-IHC tests used with references

The Dual colour break-apart FISH analysis (Vysis ALK Break Apart FISH Probe Kit; Abbott Mol Inc 06N38-020, Des Plaines, IL) was the standard diagnostic test for ALK testing approved by FDA in 2011 as companion test for the selection of patients eligible for crizotinib treatment (www.fda.gov). Other FISH test are ZytoLight® SPEC ALK/EML4 TriCheck™ Probe (ZytoVision GmbH, Bremen, Germany), Repeat free™Poseidon™ ALK break apart test (Kreatech, Leica biosystems, Nussloch GmbH, Germany) and Abnova DY EML4/ALK split FISH probe (Abnova, Taipei city, Taiwan)[1,2].

Tests are used to detect ALK expression with IHC for ALK protein expression and RNA-based-PCRs. For IHC three antibodies have been reported for the detection of ALK in NSCLC, e.g. D5F3 (Ventana), ALK1 (DAKO), and 5A4 (Abcam)[2]. The 5A4 clone seems to perform less than the D5F3[3-5]. Those staining tests can be combined with different detections systems, e.g. Novolink, Leica Biosystems, Envision Flex+, Dako, iAEP, Nichirei and OptiView amplification system.

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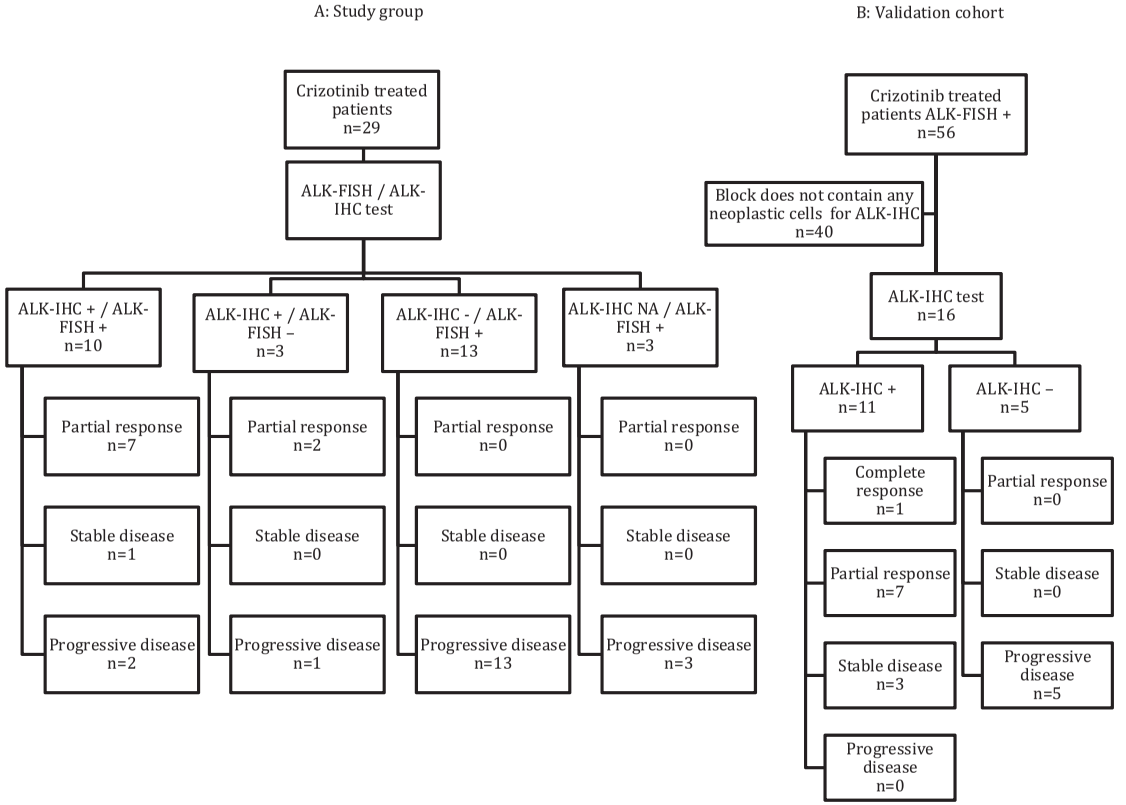
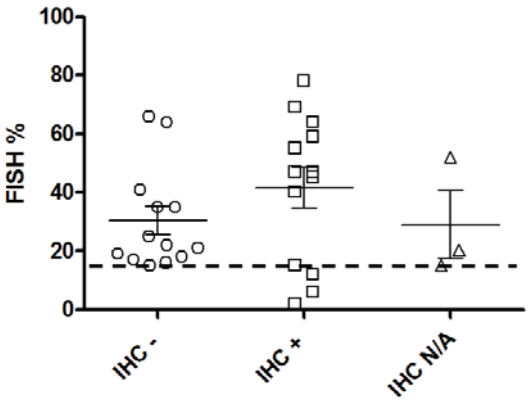
Supplementary Table 7.1. Probes used in EML4-ALK-RT-PCR[6]

Primer target	Sequence (5' to 3')
EML4 exon 1 F	CGG TCC GCT GAA TGA AGT
EML4 exon 2 F	AAG ATC ATG TGG CCT CAG TG
EML4 exon 3 F	TGG TGC AAA CAG AAA ACC AA
EML4 exon 4 F	CCC TCT TCA CAA CCT CTC CA
EML4 exon 5 F	ACG ACC ATC ACC AGC TGA AA
EML4 exon 6 F	CTG CAG ACA AGC ATA AAG ATG
EML4 exon 7 F	GTC GGC CAA TTA CCA TGT TC
EML4 exon 8 F	CTT CCG ACC GGG AAA ATA GT
EML4 exon 9 F	ACA TCC TGA CAA AAT TAG GAT TGC
EML4 exon 10 F	CCT CTA CAA CCC CAC GTC AG
EML4 exon 11 F	GCA TAT GCT TAC TGT ATG GGA CTG
EML4 exon 12 F	TTT CAC CCA ACA GAT GCA AA
EML4 exon 13 F	GAC TCA GGT GGA GTC ATG C
EML4 exon 14 F	AAG CTC ATG ATG GCA GTG TG
EML4 exon 15 F	TGT AGC AGA AGG AAA GGC AGA
EML4 exon 16 F	GTC TTG CCA CAC ATC CCT TC
EML4 exon 17 F	CCA GGA CAC TGT GCA GAT TT
EML4 exon 18 F	AGG TGG TTT GTT CTG GAT GC
EML4 exon 19 F	CCT TCC TGG CTG TAG GAT CTC
EML4 exon 20 F	CAG ATA TGG AAG GTG CAC TG
EML4 exon 21 F	ATT CCA AAT GGC TGC AAA CT
EML4 exon 22 F	AGC TGT TGC CGA TGA CTT TT
ALK exon 20 R	FAM-AGC TTG CTC AGC TTG TAC TC
EML4 intron 17 R	FAM-TTT AAT GAG TTT AAT TTT GGG
B2M F	FAM-TGA CTT TGT CAC AGC CCA AGA TA
B2M R	TGT GCA TAA AGT GTA AGT GTA TAA GCA

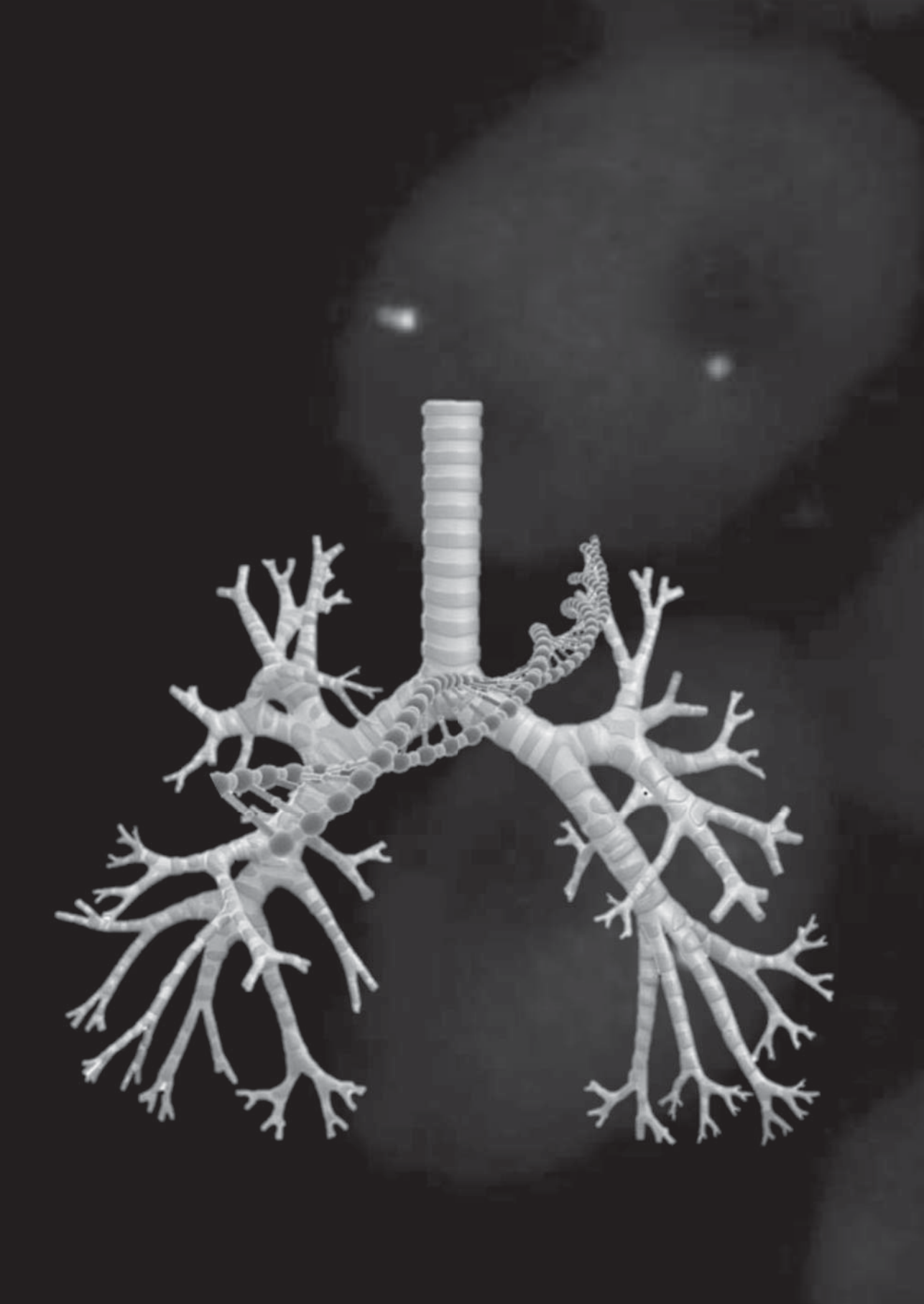


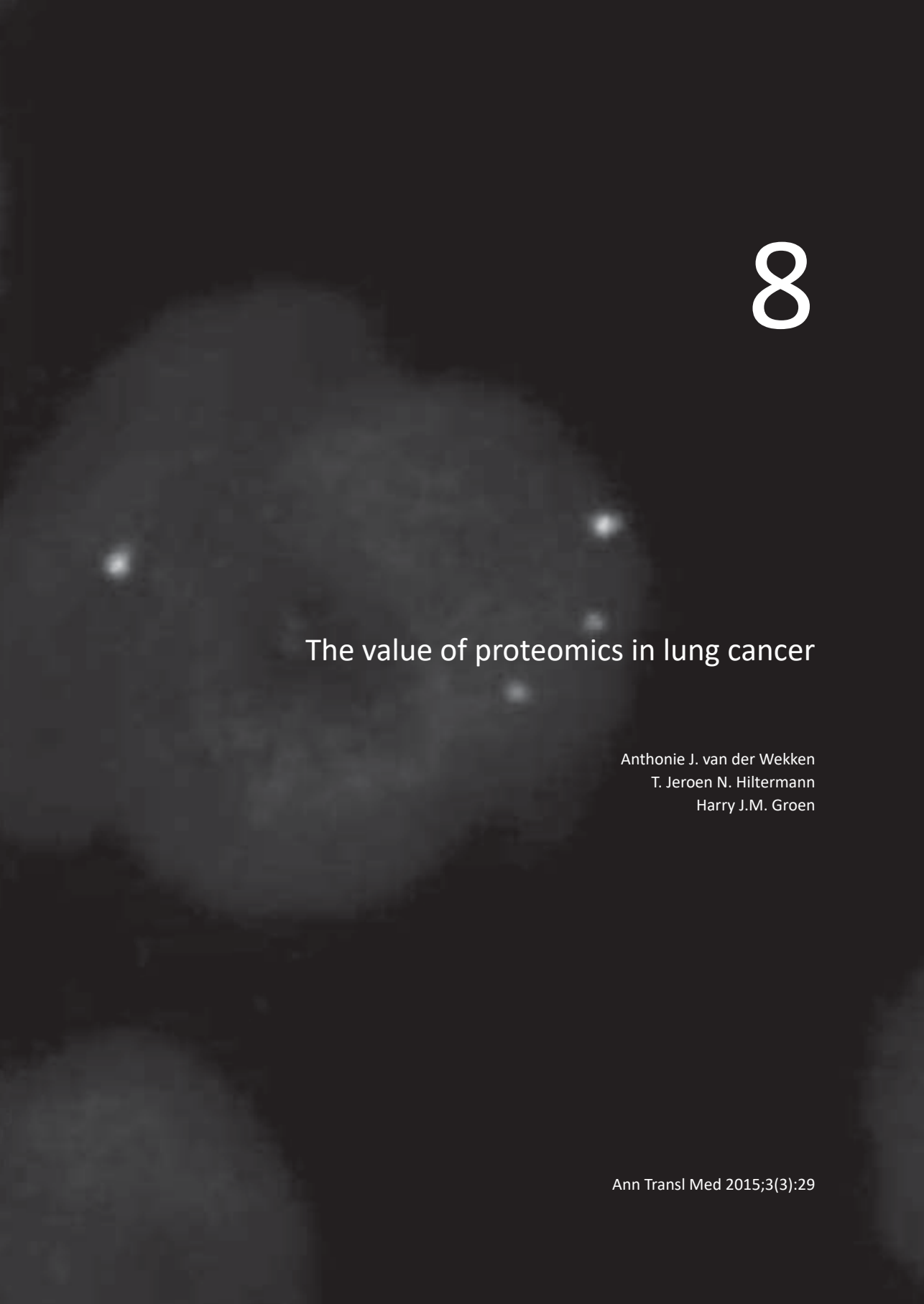
Supplementary Figure 7.1. Flow diagram of patients in study cohort and external validation.
A: Flow chart of study group treaed with crizotinib with different testing outcome, e.g. ALK-FISH positive or negative and dichotomous ALK-IHC positive or negative, divided in four groups. Response to crizotinib treatment in set below.
B: Flow chart of validation cohort, where most patients were exluded due to lack of tumour cells in blocks. Patients of whom blocks contained tumour, dichotomous ALK-IHC was performed. Response is given divided in dichotomous ALK-IHC positive and negative groups.



Supplementary Figure 7.2. Percentage of nuclei considered as ALK positive based on ALK-FISH in the screening cohort.

Dashed line: ALK-FISH cut off of 15% that determines test positivity. ALK-IHC is either positive or negative using the dichotomous test from Ventana. N/A: tumour tissue block previously used for ALK-FISH analysis that did not contain neoplastic cells anymore for dichotomous ALK-IHC.





8

The value of proteomics in lung cancer

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Ann Transl Med 2015;3(3):29

Abstract

Many studies have identified the prognostic and predictive value of proteins or peptides in lung cancer, but most failed to provide strong evidence for their clinical applicability. The strongest predictive proteins seem to be fatty acid-binding protein heart and the 8-peak mass spectrography signature of VeriStrat. When focusing on VeriStrat, a 'VeriStrat good' profile did not discriminate between chemotherapy and erlotinib. The 'VeriStrat poor' profile showed a better outcome to chemotherapy than to erlotinib. VeriStrat is a prognostic test and only the "poor profile" discriminates for the type of therapy that should be chosen. Whether it adds useful information in patients with advanced NSCLC and wildtype EGFR mutations is still doubtful. The position of the VeriStrat test in clinical practice is still not clear and we are waiting for prospective studies where biomarker tests are involved in clinical decision.

1. What do we know about proteomics in lung cancer?

Proteomics is the study of hundreds or even thousands of proteins and/or peptides in cells or organisms. Different studies have been performed to identify the prognostic and predictive value of proteins or peptides in lung cancer. Protein expression depends on transcriptional, translational and post-translational levels and can vary over a large range. Matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) and two-dimensional gel electrophoresis are commonly used techniques that detect hundreds of low-molecular weight and abundance proteins. Reproducibility and a large number of unidentified signals are known problems. More novel approaches with a better reproducibility is the high-throughput peptide sequence identification by multidimensional liquid chromatography tandem mass spectrometry that can be used in tumour tissue, pleural fluid and plasma[1,2].

In a large set of blood-derived proteins, acute phase reactant proteins are prominently present. For example macrophage migration inhibitory factor (MIF) and cyclophilin A (CyP-A) have been found in tissue, haptoglobine (HP) and a-1-antitrypsin (A1AT) have been identified amongst others as a diagnostic in serum[3,4].

Prognostic biomarkers have all been studied in tissue samples. Mostly factors predicting a poor prognosis have been found. Several markers for different types of lung carcinoma were identified. Examples are cytokeratines, heat shock proteins and annexins[5].

Predictive protein profiles have been identified as markers that can predict outcome on treatment in patients[6]. In addition other markers have been identified as being predictive, e.g. fatty acid-binding protein heart (H-FABP), for patients treated with gefitinib[7]. The other more known predictive proteomic assay is the 8-peak mass spectrography signature (VeriStrat)[8].

2. What do we learn from predictive proteomics in lung cancer?

Okano et al found in plasma in advanced NSCLC nine spots using mass spectrometry, which corresponded with nine gene products (Ig mu chain C region, Ig a-1 chain C region, SNX6, Cytoplasmic antiproteinase 3, Macrophage capping Protein, Sulfatase modifying factor 2, Glutathione S-transferase P, Ferritin heavy chain, H-FABP), in a group of patients who responded to gefitinib treatment. However, most of the patients that responded to gefitinib had an EGFR activating mutation, both in the study cohort and the validation group[7]. Therefore it seems that the identified proteins found in this study, do not have any added value to mutation analysis.

Taguchi et al. identified eight peaks (5843, 11446, 11530, 11685, 11759, 11903, 12452 and 12580 Da) using MALDI MS, that are a predictive serum markers for a good or poor response to EGFR-TKI[8]. This assay, also known as the VeriStrat essay, is under patent; therefore the identitfication of the proteins involved is not publicly known. A single-arm phase II study of erlotinib in first-line advanced lung cancer (Eastern Cooperative Oncology Group 3503) showed that patients with a 'VeriStrat good' signature had a better overall survival than patients with a 'VeriStrat poor' signature (HR 0.36; 95% CI, 0.21-0.60; p=0.001)[9]. However, in 155/239 patients mutational analysis on EGFR failed. Therefore, also this study may have been biased with activating EGFR mutations. These results were confirmed in a study by Carbone et al., who treated patients with erlotinib and bevacizumab. Here also the patients with a mass spectrometry outcome of 'VeriStrat good' had a better OS compared to the 'VeriStrat poor' group (HR 0.14; 95% CI, 0.03-0.58; p=0.007)[10]. An Italian study showed comparable results[11].

In the NCIC BR.21 trial patients with advanced NSCLC received either erlotinib or placebo. Retrospectively analysed the placebo group patients with 'VeriStrat good' signature had a far better outcome on OS compared to 'VeriStrat poor'. Both groups, good and poor, had benefited from treatment with erlotinib compared to placebo[12]. This means that VeriStrat is a prognostic biomarker, rather than a predictive marker. The prognostic value of the VeriStrat test in advanced NSCLC has been observed in studies with combinations of targeted agents both for sorafenib or bevacizumab in combination with erlotinib[13,14]. The prognostic test characteristics were further confirmed by a pooled analysis of two phase II trials (SAKK19/05 and NTR528)[15].

VeriStrat did not predict chemotherapy outcome. In a phase II study where gemcitabine was compared to erlotinib or gemcitabine/erlotinib in elderly patients, VeriStrat only was predictive for the groups who also received erlotinib in the treatment regimen[16]. A recent meta-analysis of the above mentioned studies concluded, however, after pooling the data, that VeriStrat is a predictive factor for tumour response to EGFR-TKI[17].

The PROSE study, a biomarker stratified phase III trial comparing 2nd line chemotherapy to erlotinib, added some new findings regarding VeriStrat. An OS of 9.0 months (95% CI, 6.8-10.9) was found in the chemotherapy group compared to 7.7 months (95% CI, 5.9-10.4) in the erlotinib arm. Stratifying for 'VeriStrat good' showed comparable OS between chemotherapy and erlotinib (10.9 mo; 95% CI, 8.4-15.1 vs. 11.0 mo; 95% CI, 9.2-12.9). In the 'VeriStrat poor' group a far worse outcome on treatment has been found, especially for the erlotinib treated patients (6.4 mo; 95% CI, 3.0-7.4 vs. 3.0 mo; 95% CI, 2.0-3.8).

According to the article OS results remained similar if the 14 patients with an activating EGFR mutation were excluded[18]. Therefore we can conclude that the VeriStrat is a prognostic test and only a predictive test for the VeriStrat poor profile. These patients should be treated with chemotherapy. The EMPHASIS study of ETOP was designed to explore the predictive ability of the VeriStrat signature, by testing for interaction between erlotinib vs. docetaxel and VeriStrat status using progression-free survival as primary outcome. The study was prematurely closed.

3. How should we treat patients according to predictive blood-borne biomarker?

Summarizing the data, 'VeriStrat poor' patients should not be treated with an EGFR-TKI. Patients with a 'VeriStrat good' signature have better survival outcomes independent of treatment. This implies that we could test every wild type EGFR patient with VeriStrat and treat 'the poor' profile with chemotherapy. Until further validation studies have been performed with biomarkers as clinical decision tool, there is yet no place for these biomarker tests in clinical practice.

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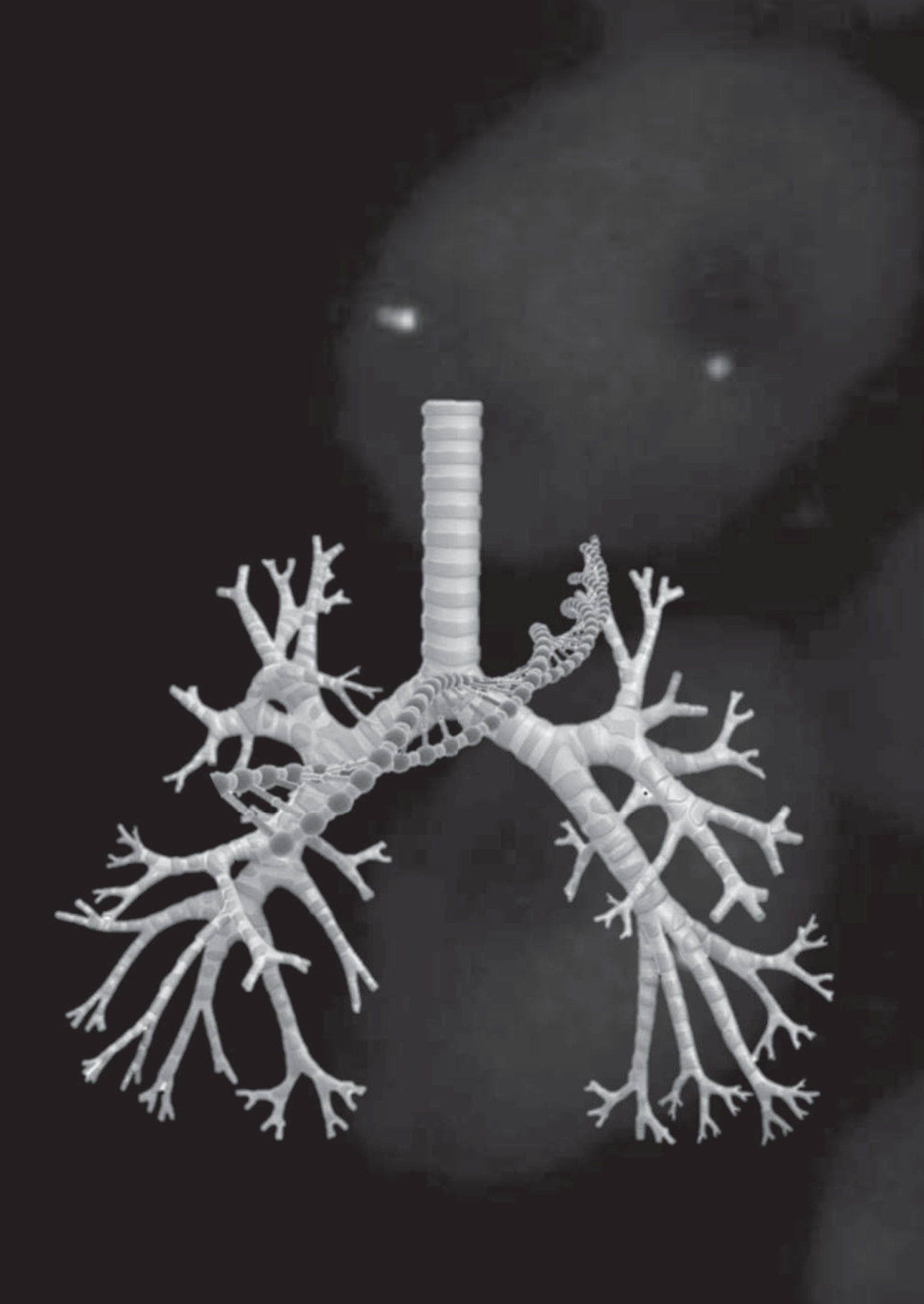

9

A novel EGFR mutation in exon 19 showed stable disease after TKI treatment

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J Thorac Oncol. 2012 Aug;7(8): e8

1. Letter to the editor

In the May 2012 issue of *Journal of Thoracic Oncology*, we were encouraged to submit clinical response data regarding epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKIs) in patients with uncommon EGFR mutations[1]. In this letter we would like to share our experience of a case with an EGFR mutation that has not been described before.

In July 2011, a 59-year old white woman was referred to our hospital with a T2aN0M1b (stage IV) adenocarcinoma of the left lung. She had carcinomatous pleuritis, carcinomatous lymphangitis, multiple lung metastases in the contralateral lung, and a distant metastasis in the seventh rib at the left side. She presented with an Eastern Cooperative Oncology Group performance score of 3. Her clinical situation was dominated by dyspnoea and hypoxemia. She was a light smoker until 1988.

Molecular analysis was performed on a formalin-fixed and paraffin-embedded cell block of pleural fluid that was acquired by ultrasound-guided fine needle aspiration. After screening using real-time polymerase chain reaction followed by high-resolution melting analysis, exon 19 was further analysed using direct sequencing and pyrosequencing (Pyromark, Qiagen, The Netherlands). A double mutation in exon 19 was found: c.2239_2240TT>CC: p.L747P.

Although the clinical relevance of this mutation is not known, we chose an initial treatment with gefitinib (at a daily dose of 250 mg). Response assessments with 6-weekly computed tomography scans revealed stable disease according to Response Evaluation Criteria in Solid Tumours (RECIST) criteria during 6 months of treatment. No significant gefitinib toxicity was observed besides a mild grade 1 rash. The patient deceased after 6 months and 1 week of gefitinib treatment without clear radiologic progression as a result of right heart failure caused by her carcinomatous lymphangitis.

Patients with mutations in exon 19 show favourable outcomes when treated with EGFR-TKI[2]. We observed stable disease upon EGFR-TKI treatment in a patient with an adenocarcinoma of the lung that harboured a p.L747P double-point mutation in exon 19.

2. References

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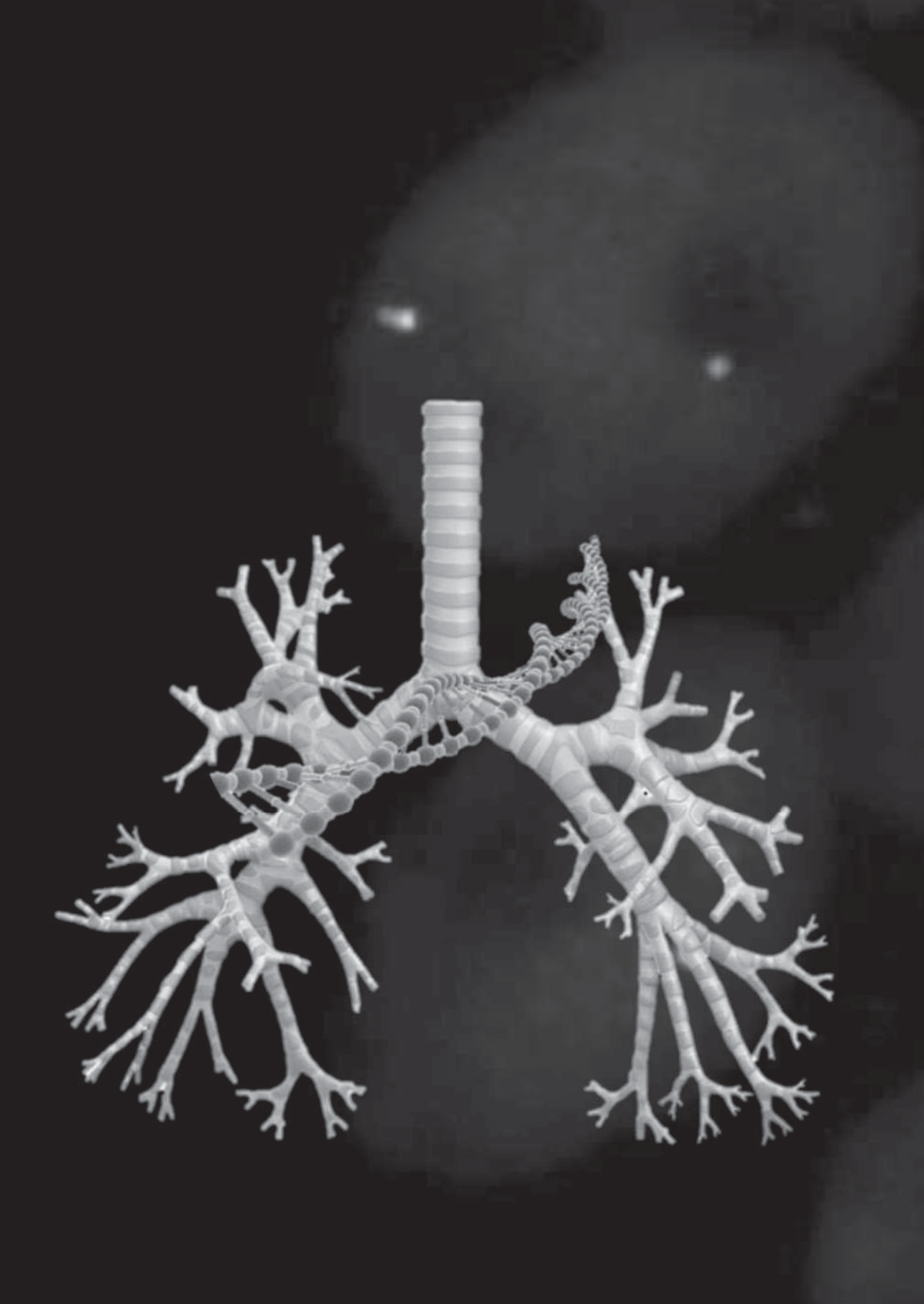

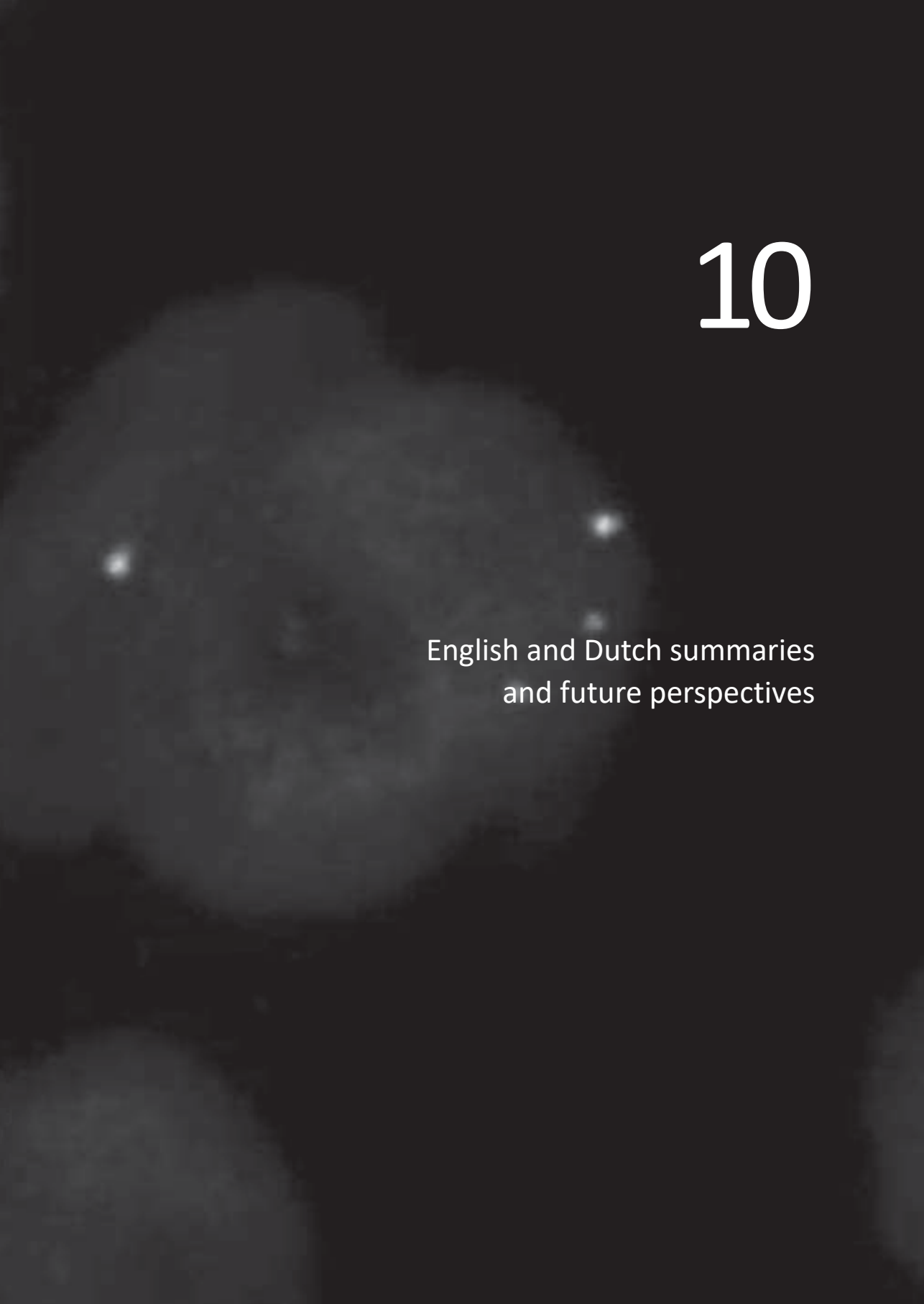

10

English and Dutch summaries
and future perspectives

1. Summary and future perspectives

1.1. Summary

In this thesis, we studied patients – mainly non-smokers – with advanced non-small cell lung carcinoma (NSCLC) with specific genomic driver aberrations. Patients were treated with tyrosine kinase inhibitors (TKIs) and most of them developed therapy resistance within several months. We have focused on efficiency of and resistance to treatment with afatinib (EGFR-TKI) in EGFR-mutation positive patients and on crizotinib (ALK inhibitor) in ALK-break positive advanced NSCLC patients.

To answer the question what we know about targeted therapy and resistance to targeted therapies, we reviewed the literature in *Chapters two and three*.

In *Chapter two* the clinically most significant mutations in lung cancer were evaluated. We also looked at sensitivity of the most commonly used approaches to detect mutations. Different next generation sequencing methods have been used, including whole genome sequencing (WGS) and whole exome sequencing (WES). Using these methods the clinically most important targetable genes identified were *EGFR*, *BRAF*, *ALK*, *ROS1*, *RET*, *HER2* and *MET*. We discussed different treatment options for each of these targets and gave a short overview of currently known resistance mechanisms. In the EGFR-mutant positive group the different resistant mechanisms were the development of T790M, MET/HER2 amplification, small cell transformation, and different treatment induced mutations in other genes. In ALK-positive patients, treatment induced ALK gatekeeper mutations, ALK copy number gain, and other gene mutations were observed.

In *Chapter three* we focused in more detail on afatinib treated EGFR-mutation positive patients and crizotinib treated *ALK*-break positive patients. We reviewed the literature on resistance mechanisms to afatinib in cell lines, mouse models, xenograft models and in patients with EGFR-mutation positive advanced NSCLC. For afatinib resistance, seven different mechanisms have been reported. For the first two mechanisms strong evidence was reported, while for the other proposed mechanisms the data mainly showed associations. The occurrence of V843I mutation in the *EGFR* gene (1) conferred resistance, whereas we and others showed that a T790M mutation did not cause resistance in patients exposed to clinically achievable doses of afatinib. *MET* amplification (2) was also reported with less sensitivity for afatinib in patients with activating EGFR mutations. In addition, resistance to afatinib has been suggested to be caused by FGFR1 amplification (3), upregulation of the IL6R/JAK1/STAT3 pathway (4), changes in the glycolytic pathway (5), Src upregulation (6) and autophagy (7).

Subsequently, an overview of resistance mechanisms in ALK-break positive patients treated with crizotinib was provided. Five different mechanisms were described. The only proven resistance mechanism in patients treated with crizotinib is the ALK gatekeeper mutation (1). Other possible off-target resistance mechanisms include upregulation of EGFR (2), KRAS mutations (3), autophagy (4) and epithelial-mesenchymal transition (EMT) (5). Most resistance mechanisms have limited evidence *in vivo* and need to be determined in patients with advanced NSCLC treated with either afatinib or crizotinib.

In *Chapter four* we determined survival of 38 afatinib treated patients (in 2nd line after first generation EGFR-TKI) with advanced NSCLC and studied resistance mechanisms in a subset of patients including 22/29 patients with a secondary T790M mutation. We did not observe survival differences in patients treated with afatinib after stratification for the T790M mutation. This was unexpected, because T790M positive tumour cells grow slower in cell lines than those without this mutation[1]. This means that, as we discussed in *Chapter three*, the T790M gatekeeper mutation does not confer resistance when patients used second line afatinib treatment in clinically achievable doses. In the first line setting, patients treated with erlotinib significantly more often developed a T790M mutation than those treated with gefitinib. These observations led to the conclusion that in the early development of tumour progression under TKIs, T790M is a prominent resistance mechanism but on afatinib, in 2nd line after first generation EGFT-TKI, other resistance mechanisms will develop that bypass T790M.

Exome sequencing was used to identify other genes with resistance-associated mutations. Thereafter, we determined in which signalling pathways these genes were involved. In a subset of patients (7/38) who became resistant to afatinib as second line treatment, we could perform exome sequencing in post-afatinib tumour biopsies. We found 284 afatinib resistance associated mutations in 68 genes. Using pathway analysis we showed that most mutations occurred in genes involved in the Wnt and PI3K-AKT pathways, indicating that afatinib resistance might depend on the signalling abrogation in those pathways. In a smaller group (3/7) with matched pre- and post-afatinib tumour biopsies, we observed 28 resistant specific mutations in six genes (*HLA-DRB1*, *AQP7*, *FAM198A*, *SEC31A*, *CNTLN*, and *ESX1*). However, those mutations did not occur in Wnt and PI3K-AKT pathway associated genes. Therefore, in this study resistance to afatinib in patients with advanced NSCLC after second line treatment was associated with mutations in multiple genes, including genes involved in Wnt and PI3K-AKT pathways. Resistance mechanisms should be investigated in each individual patient to explore possible further treatment opportunities.

In *Chapter five* resistance mechanism in crizotinib treated ALK-positive patients were studied. Matched tumour samples of 4 patients before and after crizotinib treatment were available and one non-responder with a before treatment biopsy. Whole exome sequencing analysis revealed 137 resistant specific mutations in 126 genes. These genes were significantly enriched in fourteen pathways, including four that were related to the epithelial-mesenchymal transition (EMT) process. In *Chapter three* ALK-independent resistance mechanisms have been described including advanced NSCLC without ALK gatekeeper mutations progression of disease that have been associated with EMT. So our findings are in accordance to this finding. In tumour biopsies, immunohistochemistry and cell morphology may give an indication of this transition. However this should be studied furthermore.

We extended our analysis on resistance mechanisms in *Chapter six* by studying RNA expression in tumour biopsies of ALK-positive patients. Our hypothesis was that novel treatment-induced fusion genes could be associated with resistance. We performed RNA-seq on frozen post crizotinib biopsies in three patients with a known *EML4-ALK* translocation treated with crizotinib. We observed four new gene fusions. However, these fusions were not associated with the development of resistance to crizotinib as they were present also in pre-treatment biopsies. Two of the fusion genes originated from complex rearrangements of chromosome 2, close to the ALK locus and might be attributed to this potentially genomic unstable region. In addition, we identified known gatekeeper resistance associated mutations as described in *Chapter three* in two out of three patients (p.C1156Y and p.G1269A). This emphasized not only the importance of on- and off-target resistance mechanisms but also the expression of the mutated ALK-fusion gene as a mechanism of resistance against crizotinib.

As targeted drugs bind and inhibit tyrosine kinases encoded by the mutated genes, the detection of expression of these genes may be better to select patients that benefit from targeted therapy. Most currently applied diagnostic detection methods are based on the detection of genomic alterations (mutations and rearrangements) without studying expression of these aberrant gene products.
In line with this, we hypothesized that neoplastic cells with ALK expression may be better targets than those with ALK-rearrangements. ALK fluorescence in situ hybridization (FISH) is a technique where DNA probes flanking the ALK gene locus are used to detect chromosomal breaks at the ALK locus (described in *Chapter one*). Interpretation of the FISH test is known to be difficult and presence of a DNA break is not always associated with ALK

protein expression. Aberrantly high ALK protein expression, indicative of an ALK-break, can be detected by immunohistochemistry (IHC). We performed a clinical study comparing an ALK-FISH test with dichotomous ALK-IHC in patients with advanced NSCLC in *Chapter seven*. We showed that protein expression as assessed with a dichotomous ALK-IHC test is a better predictor for outcome of tumour response and survival on crizotinib treatment, than the ALK-FISH test. Based on these results, we recommend adapting the international guidelines with dichotomous ALK-IHC as a first line standard companion diagnostic test to select advanced NSCLC patients that may benefit from anti-ALK therapy. Because ALK-IHC is easier to perform and interpret, this test could be incorporated into comprehensive standard IHC tests for the diagnosis of lung cancer, thereby selecting early in the diagnostic process patients with high ALK expression. Whether RNA-based NGS techniques will give similar insight in tumour response prediction to ALK inhibitors, will be studied in the future.

Presence of phosphorylated proteins has also been proposed to be involved in tumour response to EGFR-TKIs. Proteomics based approaches were developed and tested for their efficacy to select patients that might benefit from treatment with erlotinib. In *Chapter eight* we discussed the value of a proteomics-based test, i.e. Veristrat, for selecting the most optimal treatment of patients with wild type *EGFR*. This plasma-based test, however, did not predict outcome to treatment.

Using current diagnostic NGS techniques, we identified complex DNA aberrations and novel variants in patients with advanced lung cancers. In the UMCG, such patients are discussed in the weekly Molecular Tumour Board meetings. Treatment effects (including those observed previous in our MTB), biological plausibility of genomic aberrations and knowledge from literature in cell lines, xenografts or patient case studies are gathered. This multidisciplinary meeting discusses primarily the biological, pathological and clinical interpretation of the observed DNA aberrations in tumour and plasma but also considers IHC deviations or other relevant tests. The best treatment option for that particular patient is registered in the electronic patient files and advised to the treating physician. In *Chapter nine* we described a patient treated with gefitinib having a double mutation in EGFR, with stable disease during six months as best tumour response, which has not been described before.

1.2. Future perspectives

In this thesis we have studied different mechanism of resistance to EGFR-TKI afatinib and ALK-inhibitor crizotinib. Unfortunately, tumour samples from patients with advanced NSCLC who progressed under TKIs are scarce and often difficult to get for further analyses. Moreover, studying efficiency of TKIs on patients with rare mutations in phase III comparative studies is not feasible. Advanced NSCLC patients with activating EGFR mutations are treated with TKIs. They develop progression of disease due to tumour resistance. About half of those patients have developed a EGFR T790M resistance mutation. This mutation increases the affinity of the mutant receptor for ATP and thereby decreases the potency of gefitinib and erlotinib binding. Mutant-specific inhibitors such as osimertinib or WZ4002 irreversibly inhibit T790M mutated receptors by covalently binding to C797 at the edge of the receptor pocket. A randomized clinical trial in patients with advanced NSCLC and T790M mutation showed a high tumour response rate of 71% and about 6 months survival advantage compared to chemotherapy[2]. After this treatment a new resistance mechanism emerged, i.e. a mutation of C797 that interferes with the covalent bond of these T790M mutant-specific drugs[3]. New approaches may be the combination of allosteric inhibitors with cetuximab[4]. The first patients with this specific resistance mutation (C797S) have been encountered and may benefit from drug combinations including cetuximab and neratinib or lapatinib allosteric drugs. These three drugs are not mutant-specific but span both the allosteric and ATP site. The combination with cetuximab has to be explored.

In advanced NSCLC patients with ALK breaks and ALK expression treated with crizotinib, tumours become resistant as well. In these patients, although the ALK-break remains, ALK dependent and ALK independent resistance mechanisms occur. Especially for the group with ALK dependent mechanisms, many new drugs are under development and available in clinical studies and some are on the market already. Whether patients should be treated with drugs like ceritinib, alectinib, brigatinib or lorlatinib will be dependent on the type of gatekeeper mutation that occurs during crizotinib treatment at progressive disease[5-9]. For example the G1202R mutation is associated with resistant to crizotinib, ceritinib and alectinib, but is sensitive to lorlatinib[10]. Whether these drugs will overcome resistance in ALK-independent tumours that progressed on crizotinib treatment is not studied into detail, yet. Other drugs, e.g. X-396 and TPX-0005, have to be studied furthermore as well[11,12]. There is expectance that these drugs will overcome ALK-dependent as well as ALK-independent resistance mechanisms.

At this moment immunotherapy is the new upcoming treatment for advanced NSCLC in first line, e.g. pembrolizumab, and second line, e.g. nivolumab[13-15]. Treatment with immune modulating agents seems especially effective in tumours that have a high mutational load[16,17]. The problem of *EGFR* and *ALK* driven tumours is that these tumours in general have a low overall number of mutations and therefore have a lower probability of expressing novel tumour antigens that elicit T-cell receptor response[16,17]. Tumour responses with single agent immunotherapy are less than 25%, indicating that other treatment approaches need to be explored. In this respect, combinations of different treatments seem promising[13-15]. How we can use combinations of TKIs and immunotherapy has to be studied in more detail. However severe side effects could be a big issue here.

In a patient group, where a driver mutation is found, but no targeted therapy is available, for example in patients with mutated *KRAS*, the focus of research should be on the development of new inhibitory molecules including those effecting downstream pathways op KRAS. However, until now for most MAPK pathway mutations studies, drugs failed to show tumour responses. Whether combinations of drugs inhibiting downstream pathways will be effective should be answered in ongoing studies.
Nowadays, patients with a *BRAF* V600E mutation (in 2% of adenocarcinoma of the lung) show very good responses (response rate 63.2% [95% CI 49.3-75.6], with a median progression free survival of 9.7 months (95% CI 6.9-19.6) with a combination of dabrafenib and trametinib (BRAF and MEK inhibitor, respectively)[18]. However, it is not clear how resistance develops. Therefore, research in this patient group should focus on re-biopsy studies in treated patients to explore which treatment can be used after progression. We can probably learn from other cancer types like melanoma in which presence of BRAF V600E is more common. In those studies, it seems that BRAF V600E positive tumours can be BRAF inhibitor dependent and will stop growing after stopping BRAF inhibitors, and that rechallenge of those drugs after stopping, will give a disease control rate of 72%[19,20]. This indicates that periodical treatment is a better treatment than continuous treatment in BRAF positive patients.

On the other hand, also gene mutations are identified for which targeted therapy is available, but has not been registered yet. For example, in patients with *EGFR* exon 20 insertions, responses upon treatment with erlotinib, gefitinib and afatinib have been described[21,22]. However, in most patients treatment with these TKIs is ineffective. To predict hindrance of these TKIs in the binding pocket of the EGFR protein, we can use *in silico* 3D-modeling to predict binding efficiency and consequently predict tumour response to

mutant-specific TKIs including the more recently developed TKI like osimertinib. Using 3D-modelling approaches, we might predict response to afatinib in tumours with *ERBB2* exon 20 insertions could also be predicted by *in silico* modeling, although tumour responses to afatinib for a very limited number of insertions have been reported[23,24]. This *in silico* 3D modeling and drug binding analysis might be a very usefull complementary tool to pre-select patients who will benefit for specific treatments with specific mutations that are based on receptor hindrance.
Insight in treatment outcome of known drugs used outside the indication (off-label treatment) for rare mutations, probably has to come from registries where treatment results are collected. Different initiatives are under development in the Netherlands such as the NVALT Registry (https://www.nvalt.nl/patienteninformatie/oncologie/centra-voor-targeted-therapy). If those registries will also help molecular tumour boards to select the most optimal treatment for each patient, should be explored. Such a database should be able to help to obtain registration of current drugs for other indications.

In a large group of patients (36% of adenocarcinoma and 50% of all histologies in lung cancer) no treatable driver mutations have been identified so far. Thus, there is an urgent need for further research focusing on detection of new driver mutations or other mechanisms which are involved in the development and growth of these lung cancers[25,26]. With the targeted predictive mutation testing currently applied in the diagnostic setting, we have observed good tumour responses based on knowledge of a limited number of DNA aberrations. Examples are EGFR and BRAF mutations and ALK translocations in lung cancer. Clinical so-called basket studies have extended the number of DNA aberrations for which targeted therapy can be given, but unfortunately revealed limited efficacy today in the SHIVA trial[27]. Probably the drugs were not as specific as expected and the presence of more layers of complexity have limited the effectiveness on patient outcome. Therefore, we should study the interaction of drugs and their receptors more thoroughly and use more comprehensive DNA and RNA sequencing approaches in combination with enhanced bioinformatics tools to more accurately analyse intracellular complexity and define driver pathways. Maybe we should also include epigenetic analyses in an integrated approach in every patient with advanced NSCLC.

2. Samenvatting en toekomstperspectieven

2.1. Samenvatting

In dit proefschrift hebben we patiënten met een uitgezaaid niet-kleincellig longcarcinoom bestudeerd. De patiënten hadden longtumoren die niet gerelateerd waren aan roken en die behandeld konden worden met doelgerichte behandeling, zogenoemde tyrosine kinase remmers. Na verloop van tijd ontstaat er echter bijna altijd resistentie tegen dergelijke medicijnen. In dit proefschrift hebben we ons met name gefocust op patiënten met een DNA-mutatie in de epidermale groeifactorreceptor (EGFR), die behandeld zijn met het middel afatinib en op patiënten die behandeld zijn met het middel crizotinib vanwege een DNA-breuk in het anaplastisch lymfoomkinase (ALK).

Om de vraag te beantwoorden wat we tot op heden weten over doelgerichte behandeling en resistentie tegen die therapie, hebben we een overzicht gemaakt van de huidige literatuur in de *Hoofdstukken twee en drie*.

In *Hoofdstuk twee* hebben we de meest voorkomende mutaties (schadelijke DNA-veranderingen) beschreven. Daarnaast hebben we van een aantal testmethoden de detectiegevoeligheden op een rij gezet. We hebben de verschillende 'nieuwe generatie sequentie-analyse' (NGS) mogelijkheden, zoals het sequensen van het hele genoom (WGS) en het sequensen van de exonen (WES) beschreven. Met deze technieken worden de bouwstenen van het DNA stuk voor stuk op een rij gezet. In deze onderzoeken zijn de meest voorkomende mutaties, waar een doelgerichte therapie voor bestaat, gevonden. Het gaat hierbij om *EGFR*, *BRAF*, *ALK*, *ROS1*, *RET*, *HER2* en *MET*. Naast de mogelijke behandelopties bij deze verschillende genmutaties zijn ook de meest bekende resistentiemechanismen besproken. In de groep met een EGFR-mutatie waren extra mutaties in EGFR (T790M), amplificaties van MET/HER2, transformatie naar een kleincellig longcarcinoom en nieuwe mutaties in andere genen, de belangrijkste mechanismen van resistentie. Voor de groep met een ALK-breuk waren dat nieuwe mutaties in ALK (zogenoemde gatekeeper mutaties), toename van het aantal ALK DNA kopieën ("copy number gain") en andere genmutaties.

In *Hoofdstuk drie* hebben we een overzicht gemaakt van de bekende resistentiemechanismen in longtumoren met een EGFR-mutatie, behandeld met afatinib en met een ALK-breuk, behandeld met crizotinib. We hebben een overzicht gemaakt van de literatuur van deze twee medicijnen en daarin hebben we cellijnen, muismodellen (tumorgroei in muizen), xenograftmodellen (groei van geïmplanteerde humane tumorcellen in muizen), en patiënten met deze afwijkingen beschreven. Resistentie bij behandeling met afatinib bleek

te berusten op zeven verschillende mechanismen, waarbij het resistentiemechanisme bewezen werd voor twee van de zeven terwijl dat voor de andere vijf slechts een associatie was. De twee bewezen mechanismen zijn het ontstaan van een extra mutatie in *EGFR* (V834I), waarbij de rol van de T790M-mutatie minder belangrijk leek (1) en de amplificatie van het *MET* gen (2) waardoor de effectiviteit van afatinib op EGFR minder werd. Een associatie met resistentie tegen afatinib werd gevonden voor FGFR1-amplificatie (3), activatie van de IL6R/JAK1/STAT3 signaaltransductie (4), een verandering van de glycolyse (5), activatie van Src (6) en autofagie (7).
Voor ALK-positieve tumoren zijn vijf verschillende mechanismen beschreven. Het enige bewezen resistentiemechanisme is de ALK 'gatekeeper' mutatie (1). Andere mogelijke mechanismen zijn verhoogde expressie van EGFR (2), een mutatie in KRAS (3), autofagie (4) en epitheliaal-mesenchymale transitie (EMT; 5). Op grond hiervan blijkt dat de meeste beschreven mechanismen van resistentie niet een bewezen causaal verband hebben en dat deze mechanismen daarom nog verder bestudeerd moeten worden middels onderzoek in patiënten die behandeld zijn met afatinib of crizotinib. Daarom hebben we ons in dit proefschrift verder toegelegd op deze twee patiëntengroepen.

In *Hoofdstuk vier* hebben we de overleving van 38 patiënten beschreven die in de tweede lijn behandeld zijn met afatinib (na een eerste generatie TKI – erlotinib of gefitinib), waarbij 22/29 patiënten een resistente mutatie had in T790M. We zagen geen verschil in uitkomst van behandeling met afatinib ongeacht de aanwezigheid van de T790M-mutatie. Dat is anders dan verwacht gezien de tragere groeisnelheid van T790M bevattende tumorcellen ten opzichte van tumorcellen met andere *EGFR*-mutaties[1]. Dat betekent dat er geen effect is van een T790M-mutatie als resistentiemechanisme bij patiënten die in de tweede lijn setting worden behandeld met afatinib, zoals ook al werd bediscussieerd in *Hoofdstuk drie*. Mogelijk houdt dit verband met de te lage klinisch haalbare dosis. Daarnaast zagen we dat de patiënten die met erlotinib waren behandeld, vaker positief waren voor de T790M-mutatie dan degenen die behandeld waren met gefitinib. Deze observaties leiden tot de conclusie dat in de initiële ontwikkeling van tumorgroei gedurende therapie met doelgerichte behandeling, een extra mutatie in T790M een belangrijk resistentiemechanisme is met name bij patiënten die werden behandeld met erlotinib. In latere behandelingen lijkt het erop dat cellen met een T790M-mutatie niet bepalend zijn voor de resistentie en dat er dan dus andere resistentiemechanismen moeten zijn. In een deel van onze patiëntengroep (7/38), die resistent werden tegen behandeling met afatinib in de tweede lijn, hebben we WES verricht op biopten na therapie met afatinib.

Daarbij hebben we 284 mutaties in 68 genen gevonden, die belangrijk zouden kunnen zijn bij resistentievorming. De verschillende mutaties bleken het meest voor te komen in genen die betrokken waren bij Wnt en PI3K-AKT signaaltransductiepaden. Dit lijkt erop te duiden dat resistentie tegen afatinib berust op verstoring van de signaaltransductie in de beide genoemde paden. Bij drie van de zeven patiënten kon er ook WES worden gedaan op zowel de biopten voor als na de behandeling met afatinib. In deze groep vonden we 28 resistentiespecifieke mutaties in 6 genen (*HLA-DRB1*, *AQP7*, *FAM198A*, *SEC31A*, *CNTLN* en *ESX1*). Deze mutaties kwamen echter niet voor in de Wnt en PI3K-AKT transductiepaden. We hebben daarom geconcludeerd dat behandeling mogelijk kan liggen bij het aanpakken van de resistentiespecifieke mutaties of bij inhibitie van Wnt of PI3K-AKT signaaltransductiepaden. Het is van belang dat in elke individuele patiënt het mechanisme van resistentie bekeken wordt, zodat kan worden onderzocht wat mogelijke behandelopties zijn na een EGFR-TKI.

In *Hoofdstuk vijf* hebben we onderzoek gedaan naar resistentiemechanismen bij ALK-positieve patiënten. We hebben tumorweefsel van zowel voor als na therapie met crizotinib in vier patiënten en bij één patiënt, die direct resistent was tegen behandeling met crizotinib, hebben we materiaal van voor aanvang van de therapie bestudeerd met WES. In deze groep patiënten hebben we 137 mutaties in 126 resistentspecifieke genen gevonden. Deze 126 genen waren betrokken in 14 signaaltransductiepaden die betrokken zijn in het epitheliaal-mesenchymaal transitie (EMT) proces. Een mogelijke rol voor EMT bij resistentie tegen crizotinib werd al eerder gepubliceerd en bediscussieerd in *Hoofdstuk drie*. Daarom zullen we bij patiënten die geen 'gatekeeper' mutatie in ALK hebben, moeten kijken naar ALK-onafhankelijke resistentiemechanismen zoals EMT. In de analyse van tumorbiopten kunnen immuunhistochemie en celmorfologie helpen in onderzoek naar deze transitie. Dit zal echter nog verder onderzocht moeten worden.

We hebben daarnaast ook gekeken naar mogelijke resistentiemechanismen met behulp van RNA (2^{e} macromolecuul dat essentieel is als boodschapper voor de eiwitsynthese) expressie in ALK-positieve patiënten. Onze hypothese was dat er mogelijk nieuwe fusiegenen zouden kunnen ontstaan als mechanisme van resistentie in plaats van 'gatekeeper' mutaties. In *Hoofdstuk zes* hebben we geprobeerd met behulp van RNA sequentieanalyse deze nieuwe fusiegenen te vinden bij drie patiënten waar we voldoende direct gevroren tumormateriaal hadden van biopten die afgenomen waren na therapie met crizotinib. Bij deze analyse hebben we vier nieuwe fusiegenen gevonden. Deze fusiegenen leken echter niets te maken te hebben met geneesmiddelresistentie, omdat deze fusiegenen ook al aanwezig

waren in het biopt voor de behandeling begon. Interessant is wel dat ze meer betrokken lijken bij genomische instabiliteit van een regio op chromosoom 2, wat vlakbij het ALK-gen gelegen is. Bij deze analyse vonden we wel 'gatekeeper' mutaties in twee van de drie patiënten (p.C1156Y and p.G1269A), zoals beschreven in *Hoofdstuk 3*. Dit benadrukt niet alleen het belang van on- en off-target resistentiemechanismen, maar ook de expressie van het gemuteerde ALK-fusiegen als een mechanisme van resistentie tegen crizotinib.

Translatie van DNA naar RNA en vervolgens naar eiwit is de normale route voor het maken van eiwitten in een cel. De eiwitten zijn eigenlijk de bouwstenen van de cel en vormen de basis van alle cellulaire processen. Ze worden op basis van hun functie ingedeeld in diverse functionele subgroepen, waarvan de tyrosinekinasefamilie er één is. Doelgerichte therapieën grijpen aan op de eiwitproducten van gemuteerde tyrosinekinasen. Wanneer er geen eiwitexpressie is, zullen TKI's niet werken. Daarom was onze hypothese dat zonder ALK-expressie er geen respons op therapie zou plaatsvinden door ALK-remmers, ook al wordt er wel een ALK-breuk gevonden in het DNA. ALK-FISH (fluorescentie in situ hybridisatie) is een techniek waar DNA-probes aanhechten vlakbij het ALK-gen om breuken in het ALK-gen te detecteren (beschreven in *Hoofdstuk 1*). Interpretatie van een FISH-test is lastig en de aanwezigheid van een ALK-breuk betekent niet automatisch dat er ook ALK-expressie is. ALK eiwitexpressie kan worden onderzocht met ALK-IHC (immuunhistochemie). Om het belang van ALK eiwitexpressie te onderzoeken voor de response op therapie hebben we onze standaard ALK-FISH vergeleken met onze dichotome ALK-IHC. Deze laatste test is positief of negatief, terwijl bij de FISH-test het percentage positieve cellen een continue variabele is. De grenswaarde is op 15% breuken bepaald, omdat boven deze waarde meer tumorresponders voorkwamen. In *Hoofdstuk zeven* toonden we aan dat de dichotome ALK-IHC-score veel beter kon voorspellen of er een respons zou zijn en of er een voordeel in overleving zou zijn, dan de ALK-FISH-test dat kon. Op basis van deze resultaten hebben we geadviseerd om de huidige internationale richtlijnen te veranderen, zodat de dichotome ALK-IHC de standaardtest wordt om patiënten te selecteren die behandeld moeten worden met ALK-remmers. Omdat het om een vrij eenvoudige test gaat, kan dit worden geïncorporeerd in de standaarddiagnostiek van longkankerpatiënten met uitgezaaide ziekte. Daarmee kan vroegtijdig gevonden worden of een patiënt ALK-expressie heeft. Of het beter is om in de toekomst tumorrespons te voorspellen met behulp van NGS op basis van RNA-technieken zal verder onderzocht moeten worden.

Aangezien effectiviteit van EGFR-remming geassocieerd is met de aanwezigheid van gefosforyleerd EGFR-eiwit, zijn er 'proteomics' gebaseerde testen ontwikkeld om patiënten

te selecteren voor therapie met erlotinib. In *Hoofdstuk acht* hebben we een overzicht gemaakt van de 'proteomics' testen, zoals Veristrat, die op dit moment niet gevalideerd zijn voor de selectie van patiënten zonder activerende EGFR-mutatie voor behandeling met EGFR-remmers.

In de dagelijkse praktijk vinden we steeds vaker patiënten met tot nog toe onbekende mutaties in de tumorcellen. Dergelijke patiënten bespreken we in onze moleculaire tumorboard, om in een multidisciplinair team de best mogelijke therapie te kunnen bepalen en aan de behandelend arts te adviseren. Hierbij wordt in het overleg gebruik gemaakt van onder andere literatuur van cellijnen, xenograftmodellen en case reports. Vervolgens worden de biologische, pathologische en klinische interpretatie van de mutaties en translocaties besproken. Hierin worden testen op tumormateriaal, zoals immuunhistochemie, en plasma meegenomen, maar ook andere relevante testen. Een voorbeeld hiervan is beschreven in *Hoofdstuk negen* waarbij we twee mutaties in EGFR vonden bij een patiënte met een longcarcinoom. Behandeling met gefitinib gaf stabiele ziekte gedurende zes maanden.

2.2. Toekomstperspectief

In dit proefschrift hebben we verschillende mechanismen van resistentie bekeken bij de EGFR-TKI afatinib en bij de ALK-remmer crizotinib. Helaas is het verkrijgen van tumormateriaal na behandeling met een TKI niet altijd makkelijk en vaak is er te weinig tumormateriaal voor verdere analyse. Daarnaast is het lastig om bij weinig voorkomende mutaties goede fase III onderzoeken te verrichten. Patiënten met een vergevorderd stadium longkanker, die een activerende EGFR-mutatie hebben, worden behandeld met TKI's. Bij progressie van ziekte zien we in ongeveer de helft van de patiënten een *EGFR* T790M-mutatie ontstaan. Deze mutatie zorgt voor een toename van affiniteit van de receptor voor ATP. Hierdoor neemt de bindingscapaciteit van gefitinib en erlotinib af. Mutatiespecifieke TKI's zoals osimertinib en WZ4002 remmen T790M-gemuteerde receptoren irreversibel door een covalente binding met C797 op de rand van de receptorpocket. Een gerandomiseerde klinische studie bij vergevorderde longkankerpatiënten toonde een hoog tumorresponspercentage van 71% en een overlevingswinst van ongeveer zes maanden ter opzichte van chemotherapie[2]. Na deze behandeling zien we nieuwe resistentiemechanismen ontstaan, zoals een mutatie in C797 die de covalente binding van deze T790M-mutatiespecifieke TKI verstoort[3]. Nieuwe behandelmogelijkheden liggen in bijvoorbeeld combinaties van allosterische remmers in combinatie met cetuximab[4]. De eerste patiënten met deze specifieke resistentemutatie

(C797S) zijn gevonden en kunnen mogelijk voordeel hebben van combinaties met allosterische medicamenten neratinib of lapatinib in combinatie met cetuximab. Deze medicamenten zijn niet resistentiespecifiek, maar grijpen aan op zowel de allosterische als de ATP-bindingsplaats van de receptor. Dit zal verder klinisch onderzocht moeten worden. Bij patiënten met een vergevorderd stadium longkanker met ALK-expressie, zien we ook resistentie ontstaan. In die patiënten, zien we ondanks dat de ALK-breuk blijft bestaan, zowel ALK-afhankelijke ("on-target") als ALK-onafhankelijke resistentie ("off-target") mechanismen ontstaan. Met name voor de groep met ALK-afhankelijke resistentiemechanismen zijn er veel nieuwe middelen in ontwikkeling of al op de markt verkrijgbaar. Of patiënten moeten worden behandeld met de middelen ceritinib, alectinib, brigatinib of lorlatinib is afhankelijk van de gatekeepermutatie die gevonden wordt bij progressie van ziekte[5-9]. Zo is bijvoorbeeld bekend dat de G1202R-mutatie resistentie geeft bij de middelen crizotinib, ceritinib en alectinib, terwijl dat niet het geval is voor lorlatinib[10]. Of deze middelen ook effectief zijn bij crizotinib behandelde patiënten met resistentie berustend op ALK-onafhankelijke mechanismen is nog niet in detail bekend. Andere nieuwe medicamenten zoals X396 en TPX-0005 zullen hierin ook verder onderzocht moeten worden[11,12]. De verwachting is dat deze middelen zowel de ALK-afhankelijke als onafhankelijke resistentiemechanismen aan zullen pakken.

Op dit moment is immunotherapie de nieuwste behandelentiteit voor vergevorderd niet-kleincellig longcarcinoom. In de eerstelijn behandeling wordt dat pembrolizumab en in de tweedelijn is dat nivolumab[13-15]. Behandeling met immuunmodulerende middelen blijken met name effectief in tumoren met een hoog aantal mutaties in de tumorcellen. Hierdoor is de kans groter dat er nieuwe tumorantigenen worden gepresenteerd waardoor T-celactivatie optreedt[16,17]. Het probleem voor EGFR- en ALK-positieve tumoren is dat er over het algemeen weinig mutaties aanwezig zijn. Omdat er bij longkankerpatiënten slechts in 25% van de behandelingen met immunotherapie responsen worden gezien wanneer dit als monotherapie wordt gegeven, zullen er andere combinatietherapieën moeten worden bedacht[13-15]. Hoe combinaties van TKI's en immunotherapie vorm moeten hebben, zal eerst verder bestudeerd moeten worden. Forse bijwerkingen lijken een probleem te zijn.

In de groep waar wel een mutatie als drijfveer voor tumorgroei bekend is, maar waar nog geen doelgerichte behandeling voor handen is, zoals KRAS, zal de focus van research misschien meer verplaatst moeten worden naar het vinden van effectieve manieren voor het blokkeren van deze geactiveerde eiwitten. Tot nu toe is echter gebleken dat er geen responsen waren op therapie bij de meeste van deze MAPK-gerelateerde mutaties.

Op dit moment kunnen patiënten met een *BRAF* V600E-mutatie goed behandeld worden (response rate van 63.2% [95% CI 49.3-75.6] met een mediane progressievrije overleving van 9.7 maanden [95% CI 6.9-19.6]) met een combinatie van dabrafenib en trametinib (een BRAF- en MEK-remmer)[8]. Het probleem is echter dat bij progressie er nog geen duidelijke mechanismen van resistentie bekend zijn. Daarom is het noodzakelijk om tumorbiopten van patiënten die progressie van ziekte laten zien te bestuderen en op basis van genomische analyses te bepalen wat de beste vervolgbehandeling zou kunnen zijn. We kunnen in deze groep wellicht leren van wat bekend is bij de therapie van andere typen tumoren zoals melanomen. In die studies lijkt het erop dat BRAF-positieve tumoren een ander groeipatroon (of stopt met groeien) laten zien bij het staken van de BRAF-remmers. Een teruggeven van de BRAF-remmer bij hernieuwde groei, liet opnieuw een responspercentage zien van 72%[19,20]. Deze bevindingen geven aan dat het periodiek behandelen wellicht beter is dan continue behandelen bij BRAF-positieve patiënten.

Aan de andere kant zijn er ook DNA-mutaties waar mogelijk al een doelgerichte behandeling voor op de markt is, maar waarvoor nog geen registratie bestaat. Een voorbeeld is een EGFR-mutatie in exon 20. Hierbij zijn sporadisch responsen beschreven van behandeling met erlotinib, gefitinib en afatinib, maar helaas blijkt dat dit in de meeste gevallen geen effect heeft[21,22]. Om te begrijpen of bepaalde medicamenten wel of niet gehinderd worden door zulke DNA-mutaties maken we nu gebruik van 3D (*in silico*) modellen om effectieve binding van de medicamenten aan het gemuteerde eiwit te kunnen voorspellen. Dergelijke bevindingen worden vervolgens besproken binnen onze moleculaire tumorboard. Door deze vorm van modellering voorafgaand aan de therapie, kunnen we responsen op nieuwere therapieën zoals osimertinib mogelijk voorspellen. Het effect van afatinib zou op deze manier ook voorspeld kunnen worden bij *ERBB2* exon 20 inserties. Tumorresponsen zijn beschreven met deze therapie[23,24]. Met deze 3D-modellen kunnen we voorspellen wie we wel en niet moeten behandelen met specifieke doelgerichte behandeling in geval van complexe mutaties (meerdere mutaties die aanleiding geven tot receptorconformatieveranderingen), zodat we niet alle patiënten hoeven bloot te stellen aan medicatie waarvan onduidelijk is of het wel of niet werkt. Daarnaast is het van belang om inzicht te krijgen in behandeluitkomsten van medicatie gebruikt buiten de indicatie om (off label behandeling) bij zeldzaam voorkomende mutaties. Dit zal wellicht moeten vanuit een registratie waarin behandelresultaten worden vastgelegd. Er zijn nu verschillende initiatieven in ontwikkeling zoals de NVALT-registratie in Nederland (https://www.nvalt.nl/patienteninformatie/oncologie/centra-voor-targeted-therapy). Of dergelijke registraties

de moleculaire tumorboards kunnen helpen in het selecteren van de optimale therapie voor elk individu met een zeldzame mutatie, moet onderwerp worden van studies. Het combineren van deze gegevens zou het mogelijk moeten maken om een nieuwe registratie te krijgen voor andere indicaties dat nu voor handen is.

Omdat er in een grote groep van patiënten (36% van de adenocarcinomen en 50% van alle longtumoren) tot dusver geen mutatie kan worden gevonden die de drijfveer is achter de tumorgroei, zal er ook verder onderzoek moeten gebeuren naar het vinden van nieuwe behandelstrategieën[25,26]. Met de huidige manier van mutatietesten, die wordt gebruikt in de diagnostiek, zien we goede tumorresponsen gebaseerd op een klein aantal DNA-veranderingen. Voorbeelden hierin zijn EGFR- en BRAF-mutaties en ALK-translocaties in longkanker. Klinische zogenoemde basketstudies hebben ervoor gezorgd dat we meer doelgerichte behandelingen kunnen geven bij een groter aantal mutaties. Daarbij zien we helaas minder goede responsen op therapie, zoals beschreven in de SHIVA-studie[27]. Vermoedelijk zijn de medicamenten niet specifiek genoeg en de aanwezigheid van meerdere lagen van complexiteit zorgt voor een verminderde behandeleffectiviteit. Daarom moeten we de interactie van “targeted” medicatie met receptoren verder uitdiepen en zal de samenhang van DNA- en RNA-sequentie tot betere voorspellingen leiden door nieuwe bioinformatische mogelijkheden, zodat we de intracellulaire complexiteit en belangrijkste transductiepaden beter kunnen doorgronden. Wellicht zal analyse van cellulaire complexiteit verbeteren door bijvoorbeeld epigenetica erbij te voegen zodat we komen tot betere behandelmogelijkheden voor iedere patiënt met een vergevorderd stadium longkanker.

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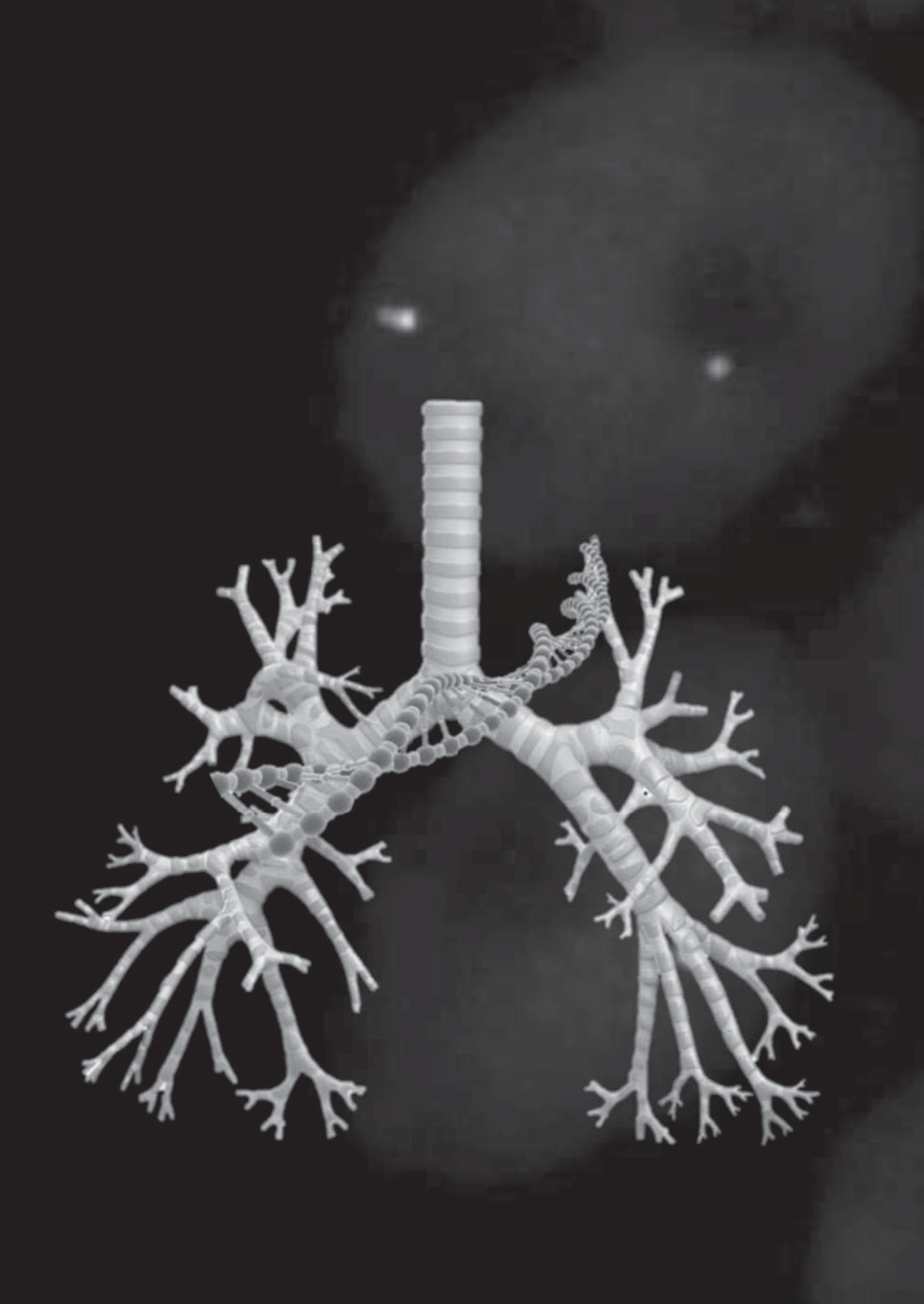

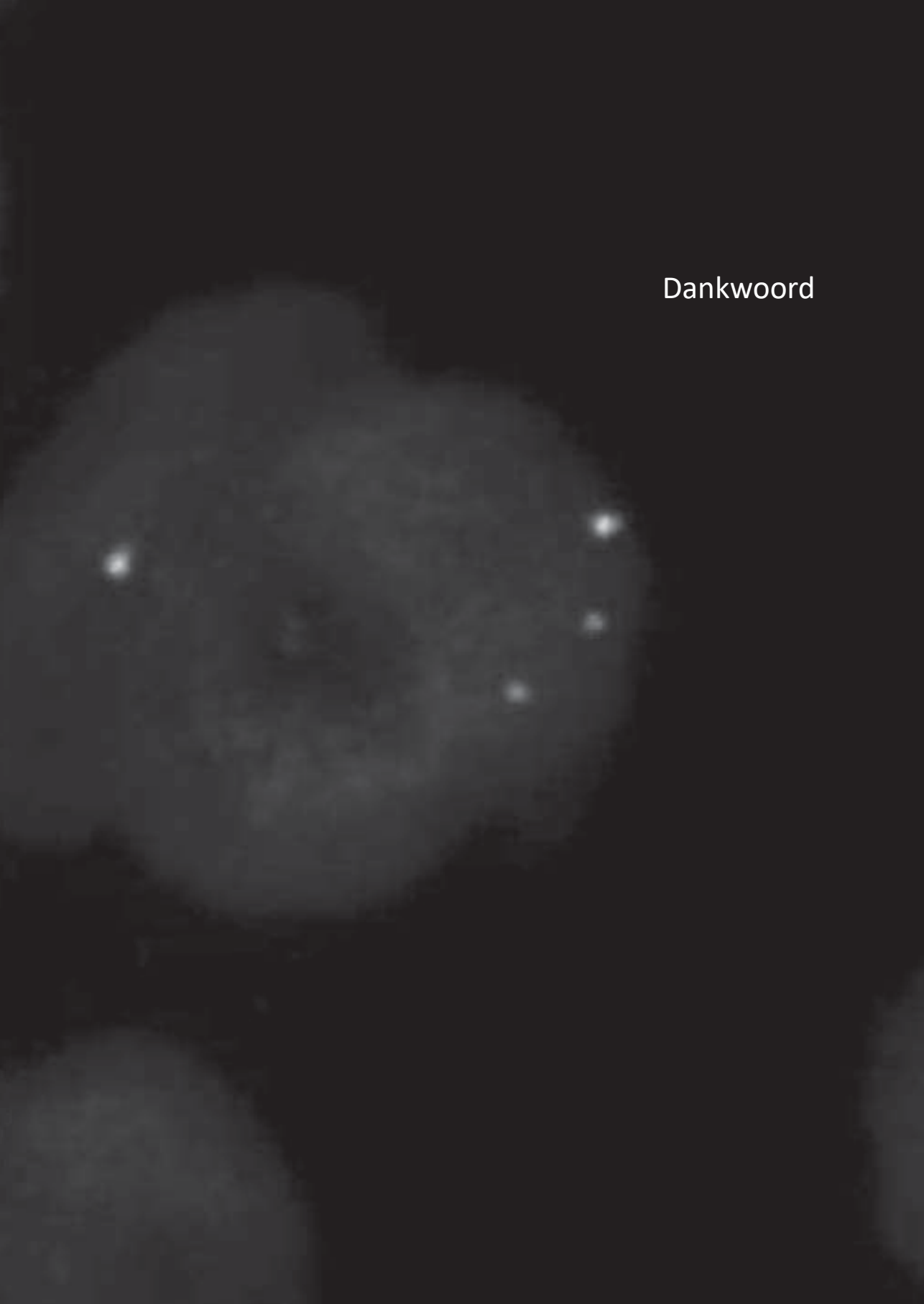

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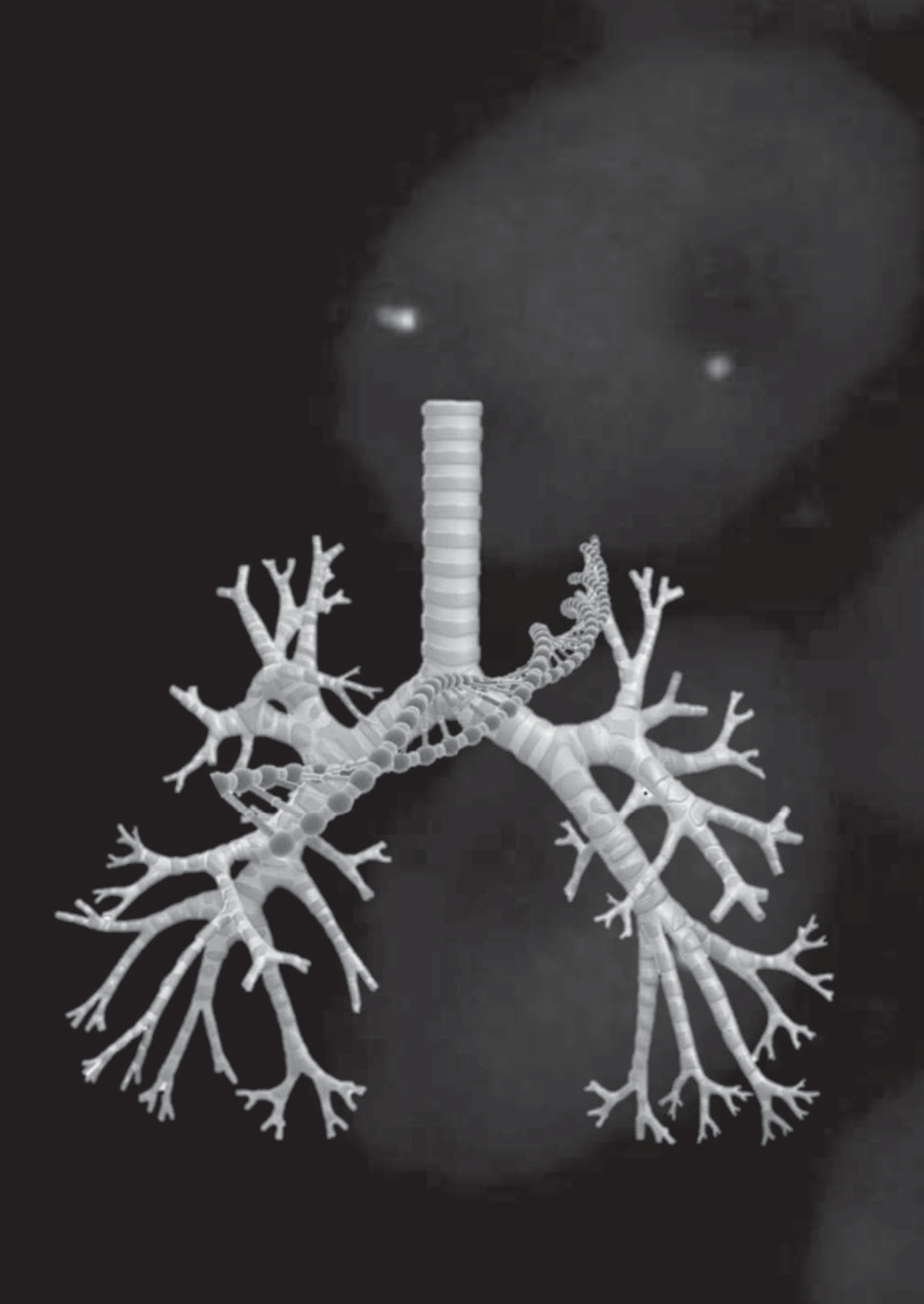

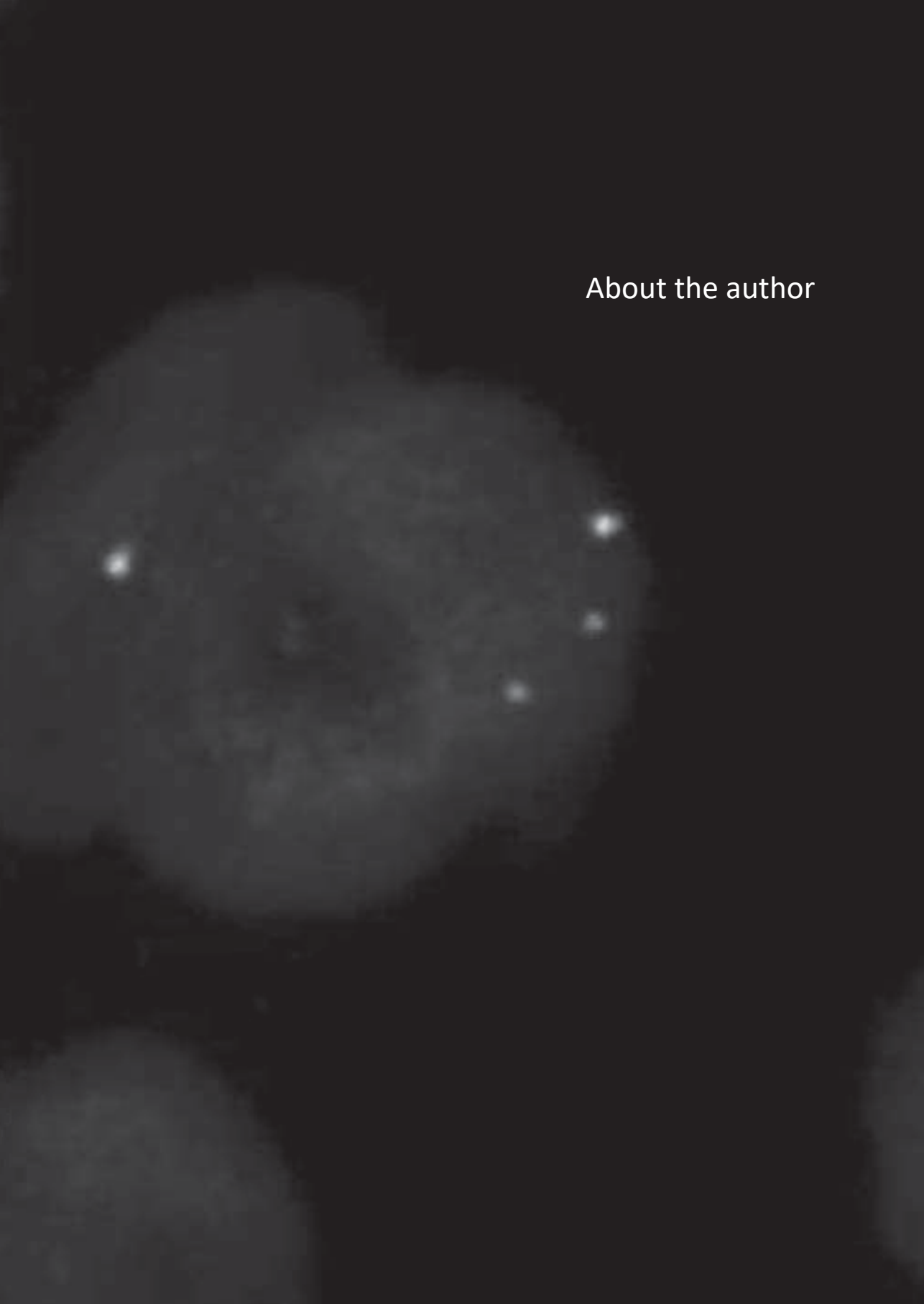

About the author

Curriculum vitae

Anthonie van der Wekken was born on December 5th 1980 in Dordrecht, Netherlands. In 1999 he finished the 'Stedelijk Gymnasium' in Leiden. From 1999 to 2005 he studied Medicine at the Erasmus University (Rotterdam, Netherlands). In December 2005 he graduated as a medical doctor.

At the beginning of 2006 he started his specialisation to become chest physician at Isala (Zwolle, Netherlands) under supervision of Dr. Jan Willem van den Berg. Since February 2012 he was registered as chest physician. For 3 months he has worked at Isala and afterwards started to work in the University Medical Centre Groningen (Groningen, Netherlands). Now he is responsible for the medical care of pulmonary oncology patients. He also is responsible for pulmonary endoscopy, especially for ultrasound guided biopsies and fine needle aspirations, e.g. transthoracic ultrasound, endoscopic ultrasound (EUS) and endobronchial ultrasound (EBUS).

Anthonie lives with his wife Alies van de Wekken – van Dieren and their four children, Thijs (2008), Noa (2010), Lars (2011) and Niels (2015). They are expecting a baby next September.

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