Genomics of lung cancer
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Chapter 5B

Genomic aberrations in crizotinib resistant lung adenocarcinoma samples by transcriptome sequencing

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University of Groningen, University Medical Center Groningen, ¹Department of Pathology and Medical Biology, ²Department of Pulmonary Diseases, ³Department of Genetics, the Netherlands

Provisionally accepted, PlosONE
Abstract

Objectives: Although most ALK-break positive non-small cell lung cancer (NSCLC) patients initially respond to crizotinib, resistance occurs inevitably. So far none of the studies focused on fusion genes as a possible mechanism to explain the resistance. In this study we aimed to identify fusion genes in crizotinib-resistant tumor samples.

Materials and Methods: Re-biopsies of three patients were subjected to paired-end RNA sequencing to identify fusion genes using DeFuse. The IGV browser was used to determine presence of known resistance-associated mutations. Sanger sequencing and digital droplet PCR were used to validate fusion genes and mutations.

Results: ALK fusion genes were detected in all three patients with EML4 being the fusion partner. Patient #1 contained, in addition, one other fusion gene, i.e. NRG1-RBPMS, but this fusion gene did not contain a predicted open reading frame. Patient #3 contained three additional fusion genes, of which two were derived from the same chromosomal region as the EML4-ALK fusion. A predicted ORF was identified only in the CLIP4-VSNL1 fusion product and not in the other two fusion genes. Patient #2 did not contain any additional fusion gene. Fusion genes were detected in the resistant tumors and primary tumors by qRT-PCR. Analysis of the RNA sequencing data revealed ALK gatekeeper mutations in two patients. These two mutations were not detected in primary tumor samples.

Conclusion: Fusion genes identified in our study are unlikely to be involved in crizotinib resistance. ALK gatekeeper mutations were identified in resistant tumor samples of two patients. In one patient, the mechanism of the resistance remained unclear.
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 the EML4-ALK fusion 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 tumor growth indicating transforming activity of the EML-ALK fusion protein\(^3\). ALK rearrangements have thereafter 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 including the tyrosine kinase activity domain of ALK\(^7\).

Despite an initial favorable response to crizotinib, patients inevitably acquire resistance due to selective pressure of the 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 mutations in the ALK kinase domain, such as C1156Y, L1196M and G1269A, and ALK copy number gain\(^8,9\). 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 resistance mechanisms to the ALK-TKI in cell lines\(^10\).

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 to detect fusion genes and validated breakpoints and presence in resistant and primary frozen samples. In addition, we used the RNA sequencing data to determine presence of crizotinib resistance-associated mutations in EGFR, KRAS and ALK genes.
Materials and Methods

Patients and tumor samples

Three crizotinib treated lung adenocarcinoma patients with a FISH-positive ALK break (Vysis LSI ALK Break Apart FISH Probe Kit, Abbott Molecular Inc., Des Plaines, USA) in >15% of the tumor cells were selected for this study based on the availability of frozen tissue of the crizotinib-resistant samples (Table 1). In addition, formalin fixed paraffin embedded (FFPE) tumor tissue samples were available before and after crizotinib treatment. A normal lung tissue sample was used as control for the RT-PCR. All procedures and protocols were performed according to the guidelines for good clinical practice and after informed consent.

Fluorescence in situ hybridization (FISH)

FISH was performed using the ALK dual color break probes (Vysis LSI ALK Break Apart FISH Probe Kit, Abbott Molecular Inc., Des Plaines, USA) following a standard protocol. 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). Slides were analyzed 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.

Immunostaining

ALK staining was done on 3 micron FFPE tumor tissue sections, using ALK rabbit monoclonal antibody clone D5F3 (Roche, Basel, Switzerland) in VENTANA BenchMark ULTRA according to the manufacturer’s protocol (Ventana, Tucson, Arizona).
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 RNasy 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 gDNA Miniprep 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.

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). DeFuse (v.0.6.1) was used to identify putative fusion gene products. DeFuse maps the reads to the reference genome using an automated process which involves SAMtools, bowtie, BLAT, and GMAP. Based on the DeFuse output, three exclusion criteria were used; 1) probability of the predicted fusion transcripts <0.95; 2) fusion transcripts derived from a putative read-through transcript; 3) probability that fusion transcripts map to an enriched expressed sequence tags (EST) region >0.95. Of the fusion genes that pass these criteria we manually screened split and spanning reads using a custom track containing the fusion gene sequence files from DeFuse using the IGV browser. As a last step, we inspected whether both sides of the fusion breakpoint were correctly mapped or derived from intergenic regions using the University of California Santa Cruz (UCSC) genome browser (Supplementary Figure S1). Predictions of the presence of an ORF in the fusion products were obtained from DeFuse.

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 MgCl₂ (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,
Identification and validation of mutations in \textit{ALK}, \textit{EGFR} and \textit{KRAS} gene

For each patient the RNA-seq bam file, generated by RSEM (1.2.9) was uploaded into the IGV browser. All exons of \textit{ALK}, \textit{EGFR} and \textit{KRAS} genes were visually screened for the presence of known resistance-associated mutations. To validate mutations, 50ng of DNA was amplified as described above using primers designed with Clone Manager Suite (Sci-Ed Software, Morrisville, USA) (Supplementary Table S1). 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 \textit{ALK} mutations was also validated by PCR and Sanger sequencing on 10ng of cDNA using primers that specifically amplify the \textit{EML4-ALK} breakpoint region.

Validation of \textit{ALK} mutations by droplet digital PCR (ddPCR)

Mutant and wild type ddPCR primers and probes to detect C1156Y and G1269A \textit{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 analyzed by Quantasoft software version 1.6.6 (Quantasoft, Prague, Czech Republic). In addition to the primary and resistant tumor samples, 10 normal 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 resistant tumor samples.

Results

Patients

The three patients, aged 27 to 56 years, were all tested positive for ALK IHC before crizotinib treatment (primary) and at disease progression (resistant). All three patients were ALK FISH positive both before crizotinib and at disease progression. Patient #1, #2 and #3 showed a partial response (PR) with progression free survival (PFS) of 7.0, 9.5 and 15.9 months, respectively. Only patient #3 showed extra ALK copies in the diagnostic FISH analysis (Table 1 and 2).

Table 1: Patients’ characteristics and fusion products detected in crizotinib resistant tumors.

<table>
<thead>
<tr>
<th>Pat.</th>
<th>Age</th>
<th>Smoking</th>
<th>Tumor response (PFS in months)</th>
<th>Samples</th>
<th>Tumor (%)</th>
<th>No. of predicted gene fusions</th>
<th>High confidence gene fusion</th>
<th>Predicted ORF</th>
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<td>#1</td>
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<td>None</td>
<td>PR (7.0)</td>
<td>Res-1</td>
<td>90</td>
<td>33</td>
<td>EML4-ALK NRG1-RBPSM5</td>
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<td>#2</td>
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<td>Current</td>
<td>PR (9.5)</td>
<td>Res-2</td>
<td>70</td>
<td>37</td>
<td>EML4-ALK</td>
<td>Yes</td>
</tr>
<tr>
<td>#3</td>
<td>42</td>
<td>None</td>
<td>PR (15.9)</td>
<td>Res-3</td>
<td>90</td>
<td>68</td>
<td>EML4-ALK CLIP4-VSNL1 MCDF2-CLIP4 CPSF6-RBMS2</td>
<td>Yes No No</td>
</tr>
</tbody>
</table>

PFS is progression free survival; Res is crizotinib resistant tumor; ORF: Open reading frame.

Detection of fusion products

A total of 19.9, 19.9 and 28.9 million reads were aligned for resistant tumor samples of patient #1, #2 and #3, respectively. Seven fusion gene products were identified in these three tumor samples, including an ALK fusion gene in each patient (Table 1 and Supplementary Figure S1). The fusion partner was EML4 in all three patients according to the DeFuse analysis. The breakpoint was in intron 20 of the EML4 gene in patient #1 and intron 6 of the EML4 gene in patients #2 and #3. The EML4 gene was fused to exon 20 of the ALK gene in all three patients.
In patient #1 one additional fusion gene, i.e. \textit{NRG1-RBPMS}, was detected but this fusion had no predicted ORF according to DeFuse. In patient #2, no additional fusion products were identified. Patient #3 contained in addition to \textit{EML4-ALK}, three fusion genes, one with and two without predicted ORFs. Two of the fusion genes (\textit{CLIP4-VSNL1} and \textit{MCFD2-CLIP4}) were the result of multiple genomic aberrations at the \textit{ALK} gene region on chromosome 2 (Figure 1A). Both fusion products involved the \textit{CLIP4} gene mapping 8kb downstream of the \textit{ALK} gene. In one fusion transcript, exon 14 of the \textit{CLIP4} gene was fused to exon 2 of the \textit{VSNL1} gene, resulting in a fusion transcript with a predicted ORF. In the second fusion transcript, exon 15 of the \textit{CLIP4} gene was fused to the non-coding exon 1 of the \textit{MCFD2} gene. This fusion did not have a predicted ORF.

\textbf{Table 2:} Summary of the diagnostic FISH and immunostaining results and the transcriptome analysis

<table>
<thead>
<tr>
<th>Pat.</th>
<th>Primary tumor</th>
<th>Resistant tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\textit{ALK} FISH (%)</td>
<td>\textit{ALK} IHC</td>
</tr>
<tr>
<td>#1</td>
<td>&gt;15</td>
<td>+</td>
</tr>
<tr>
<td>#2</td>
<td>&gt;50</td>
<td>+++</td>
</tr>
<tr>
<td>#3</td>
<td>&gt;15</td>
<td>+</td>
</tr>
</tbody>
</table>

WT: Wild type; *Presence of mutated and unmutated \textit{EML4-ALK} sequencing reads suggests gain of an \textit{EML4-ALK} fusion gene (See discussion).

\textbf{Validation of the fusion products by RT-PCR}

\textit{EML4-ALK} fusion transcripts were confirmed by RT-PCR in resistant tumor samples of patients #1, #2 and #3 (Figure 1B). We next validated the two fusion products originating from chromosomal aberrations at the \textit{ALK} genomic region identified in patient #3. A PCR product of the expected size was observed for both fusion genes in the frozen biopsy of the resistant tumor samples (Figure 1C). Sanger sequencing of these RT-PCR products confirmed the expected sequence consistent with the prediction of DeFuse. Next, we evaluated whether these fusion transcripts were also present in the primary FFPE tumor samples of these patients. FFPE samples of the resistant tumors were included as positive controls. The \textit{CLIP4-VSNL1} and \textit{MCFD2-CLIP4} fusion transcripts were detected in the primary and resistant tumor samples (Figure 1D).
Resistance to crizotinib in lung adenocarcinoma

Figure 1: Schematic representation of the fusion gene products that clustered at the ALK gene locus and validation of the fusion products using RT-PCR. A) Three fusion products clustered at a 25Mb genomic region including the ALK gene locus in patient #3. 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 resistant tumor samples. C) Validation of two novel fusion genes in frozen resistant tumor samples. D) Detection of the fusion genes in FFPE samples of resistant samples and analysis of the primary tumor samples. Norm: Normal lung tissue; Prim: Primary tumor sample; Res: Resistant tumor sample; Neg: Negative control.

Identification and validation of mutations in ALK, EGFR and KRAS

Mutations in ALK, EGFR and KRAS have been reported to confer resistance against crizotinib. Analysis the paired-end RNA sequencing data revealed an ALK gatekeeper mutation, i.e. p.C1156Y (NM_004304.3:c.3467G>A) in patient #1 in 57% of the reads. No mutations were observed in the EGFR and KRAS genes. Sanger sequencing of the RT-PCR product using primers specific for the EML4-ALK fusion gene confirmed presence of both wild type and mutant EML4-ALK fusion transcripts consistent with the RNA sequencing data (Figure 2A). This suggests that the duplication event occurred before gain of a gatekeeper mutation in one of the two ALK break copies (Table 2). In patient #3, an ALK gatekeeper mutation was observed in 100% of the RNA sequencing reads, i.e. p.G1269A (NM_004304.3:c.3806G>C). Sanger sequencing confirmed presence of the
mutations at the genomic DNA level in the resistant tumors of both patients (Figure 2B).

Figure 2: Detection of ALK gene mutations in resistant tumor samples by RNA-seq and validation in resistant and primary tumor samples by Sanger sequencing and ddPCR. A) RNA-sequencing reads of the two gatekeeper mutations. Grey bars show the wild type positions, the colored bar indicates the mutant position. The number of wild type and mutant reads were 56/75 for patient #1 (c.3467G>A) and 0/25 for patient #3 (c.3806G>C)(Top). RNA Sanger sequencing in the resistant tumor sample of patient #1 confirmed presence of one wild type and one 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 resistant and primary tumor samples. C: ddPCR results of the primary and resistant tumor samples of patient #1 and #3. Number of positive droplets for mutant and wild type alleles is written in each gate of the scatter plots. Fractional abundance for the mutant allele was 26% and 19.8% in the resistant tumors of patients #1 and #3, respectively. Prim: Primary tumor sample; Res: Resistant tumor sample.
Analysis of the ALK gatekeeper mutations in the primary tumor samples of patients #1 and #3 by Sanger DNA sequencing revealed no mutations. To exclude presence of a minor clone with the ALK gatekeeper mutation in the primary tumor samples we also performed ddPCR on the resistant and primary tumors. In the resistant tumors, the fractional abundance of the corresponding mutant alleles was 26% and 19.8% in patients #1 and #3, respectively. In the primary samples these mutations were not detected (Figure 2C).

Discussion

ALK-break positive NSCLC patients respond to crizotinib in over 60% of cases, but after 9 to 12 months drug resistance will inevitably develop. Several groups have identified gatekeeper mutations in the ALK gene or mutations in KRAS and EGFR in re-biopsies as mechanisms of the resistance. In our re-biopsy study we focused on detection of novel fusion products in tumor samples of three patients who developed resistance to crizotinib.

In patient #1, we confirmed presence of the EML4-ALK fusion gene in the resistant re-biopsy taken from a patient with a growing tumor under crizotinib. No additional fusion genes with a predicted ORF were identified in this patient. The ALK C1156Y gatekeeper mutation was identified in approximately half of the RNA-seq reads in this patient. This suggests that the ALK gatekeeper mutation was gained after duplication of the EML4-ALK fusion or that the mutation is present only in a proportion of the tumor cells, while being wild type in the other tumor cells. The alternative explanation, i.e. gain of de novo fusion gene in combination with gain of an ALK gatekeeper mutation on one of the two fusion genes seems unlikely. The mutation was not present in the primary tumor using the sensitive ddPCR (Table 2). In patient #3, we confirmed the presence of the EML4-ALK fusion gene in the re-biopsy. Three additional fusion genes were detected, of which one had a predicted ORF. However, this fusion gene was also present in the primary tumor sample, and thus not treatment induced. In addition, we observed the G1269A ALK gatekeeper mutation in the re-biopsy of the resistant tumor, which was not present in the primary tumor sample using ddPCR. Gain of a gatekeeper mutation most likely caused the crizotinib resistance in patients #1 and #3. 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\(^9\), \(^{18}\). 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 #1 may be due to the combination of different resistant mechanisms, the milder C1156Y gatekeeper mutation and the potential gain of the EML4-ALK fusion duplication. Moreover, based on the normalized RNA sequencing reads, this patient also showed a 2 to 3 fold higher expression level of the ALK fusion gene as compared to the two other patients. Thus, despite the less effective gatekeeper mutation, gain of ALK copy and the higher expression level might also have contributed to the short PFS.

In patient #2, we confirmed presence of the EML4-ALK fusion gene in the re-biopsy sample. No additional fusion genes were identified. We did not find ALK gatekeeper 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 #1 and we did detect the EML4-ALK fusion gene, it seemed unlikely that we failed to detect other fusion genes. Moreover, we found no evidence of increased expression of ALK or EGFR in the RNA sequencing data (results not shown). Other currently unknown ALK-independent resistance mechanisms might have been induced in this tumor sample.

A number of studies have investigated mechanisms of resistance to crizotinib in post-treatment tumor samples of NSCLC patients. ALK gatekeeper mutations were the most commonly observed aberrations identified in re-biopsies of 16 out of 51 (31\%) patients\(^1\), \(^9\), \(^{18-21}\). We detected ALK gatekeeper mutations in 2 of the 3 patients. ALK gain has been reported as resistance mechanism in 4 out of 36 (11\%) patients\(^1\), \(^9\), \(^{20}\). We observed EML4-ALK RNA-seq reads with and without the ALK mutation in patient #1. This might indicate duplication of the fusion gene in one of the two EML4-ALK alleles. Of the 36 patients studied for both ALK mutations and ALK gain, only one case was positive for both, similar to our patient #1.

In patient #3, two novel fusion gene products (one with and one without a predicted ORF) involving the CAP-GLY domain containing linker protein family, member 4 (CLIP4) gene were present in both the primary and resistant tumor samples. Given the gain of a functionally confirmed ALK gatekeeper mutation, it
seems unlikely that this fusion is associated with resistance to crizotinib. Moreover, this fusion product was already present in the primary tumor sample. The clustering of three fusion gene products within the ALK gene region suggests that this genomic region is an instable region in NSCLC. The frequent loss of (part of) the short arm of chromosome 2 (2p14-16, 2p23.3 and 2p24.3) as observed in NSCLC is consistent with this region to be susceptible to chromosomal breaks\textsuperscript{22-23}.

The question is whether other fusion transcripts may be of clinical importance but so far none have been described. The presence of ALK fusion variants have yet not been described as a cause for the heterogeneity in tumor response to ALK inhibition. Besides crizotinib and ceritinib that both show high tumor response rates, next generation ALK inhibitors such as alectinib, brigatinib (AP26113) and PF-06463922 are in development and show high response rates in diverse resistant 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 against L1196M, F1174L, G1269A but not against S1206Y. PF-6463922 is active in all previous mutant forms\textsuperscript{24-27}.

In conclusion, we identified four novel gene fusion products in two of the three crizotinib resistant re-biopsies. In one patient, both copy number gain and an ALK gatekeeper mutation were present as resistance mechanisms and in another patient an ALK gatekeeper mutation could explain resistance to crizotinib. In the third patient, the putative ALK-independent resistance mechanism remained unclear, but it is unlikely that the resistance has been caused by novel fusion genes.
References


**Supplementary information**

**Supplementary Table S1:** List of primers for detection of fusion transcripts and ALK mutations in frozen/FFPE samples.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence</th>
<th>DNA/RNA</th>
<th>Used on</th>
<th>Annealing (°C)</th>
<th>Product size (bp)</th>
<th>Transcript Ensembl ID</th>
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<td>5’-ATGGTTCAGTTGGAGGTGTG-3’</td>
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**Supplementary Figure S1:** Filtering steps applied to remove false positive fusion transcript predictions. After detection of potential fusions by DeFuse, we excluded predicted fusion genes based on three DeFuse criteria, probability less than 0.95, read through transcripts and fusion products located in EST enriched regions with probability of more than 0.95. In the last step, split and spanning reads were checked using the IGV browser on a custom track. The fusion genes without break split reads were removed. The remaining fusions have been inspected to determine if both sides of the fusion breakpoint were correctly mapped and not derived from intergenic regions using UCSC browser. A total of 19.9, 19.9 and 28.9 million aligned reads were obtained from transcriptome sequencing of the resistant tumor samples of patient #1, #2 and #3, respectively.