Histological and molecular plasticity of ALK-positive non-small-cell lung cancer under targeted therapy: a case report

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Abstract With medical progress in cancer therapy, tyrosine kinase inhibitors (TKIs) became a standard of care for many cancer types. But the broad range of possible targeted therapies was accompanied by a plethora of potential resistance mechanisms, of which many have still to be identified. Here, we present the case of a patient with an EML4–ALK translocated non-small-cell lung cancer treated with four different TKIs. Her tumor developed not only a well-known ALK–TKI resistance mutation but also underwent a histological transformation from adenocarcinoma to squamous cell carcinoma. To confirm a shared monoclonal origin of the phenotypically different tumors, a phylogenetic reconstruction was conducted: This revealed a cluster of mutations including NFE2L2, KMT2D, and MLH1, which are possible triggering events for the transformation.

INRODUCTION Non-small-cell lung cancer (NSCLC) is classified based on the World Health Organization’s criteria of light microscopy and immunohistochemistry (IHC). It includes the two main subtypes lung adenocarcinoma (ADC) and squamous cell carcinoma (SqCC). Comprehensive molecular workup in routine molecular diagnostics does not only allow the identification of well-established targetable driver alterations like EGFR and BRAF mutations or fusions of ALK, ROS1, RET, and NTRK1/2/3, but it also can aid in differentiating two independent primary tumors from a primary tumor and a subsequent relapse. EML4–ALK translocations
are well-known driver alterations in ADC but rarely reported in SqCCs (Dragnev et al. 2014). Here, we describe the evolution of an ALK-positive lung tumor under tyrosine kinase inhibitor (TKI) treatment from an ADC to a SqCC, supporting the suggested alternative resistance mechanism of a recent study (Park et al. 2019).

RESULTS

Case Presentation

We present the case of a histological and genetic transformation to an EML4–ALK-positive ADC to a SqCC under therapy over a period of 5 yr (Fig. 1A). A 49-yr-old female patient, ex–light smoker (5 pack years) since 2015, was first diagnosed with an EML4–ALK translocated pulmonary ADC in December 2016, based on an endobronchial biopsy of the left main bronchus. Further, initial diagnosis revealed brain metastases, pleural infiltration, and pleural effusion, but no other distant metastases. Rebiopsies following progress were taken in 2019, 2020, and 2021 as computed tomography (CT)-guided pleural biopsies from the fourth posterior intercostal space on the left, the left dorsal side, of a lesion from the fifth to eighth rib on the left side, respectively. The first two biopsies (2016, 2019) were diagnosed consistently as ADC, supported by immunohistochemical expression of TTF-1 and CK7 (Fig. 1B). Apart from single cells the tumor did not show any expression of p63, a marker commonly used to assess squamous differentiation (Supplemental Fig. S1). However, in the two later biopsies, ADC could not be diagnosed, as the tumor cells exhibited clear and solely features of a SqCC with evident keratinization and expression of CK5/6 and p63 but absence of immunoreactivity for TTF-1 and CK7. In all four biopsies tumor cells did not show any PD-L1 expression.

Treatment

First-line treatment started in January 2017 with crizotinib (2 × 250 mg/d). Upon isolated central nervous system (CNS) progression with diffuse brain lesions 2 mo later, whole brain radiotherapy was conducted (10 × 3 Gy). Because of further CNS progression, therapy was switched to alectinib (2 × 600 mg/d) in June 2017, which led to a partial intra- and extracranial remission. In June 2018, stereotactic radiotherapy (6 × 5 Gy) was administered because of oligoprogression in the brain. In April 2019, diffuse tumor growth in the left lung and pleura led to a rebiopsy that revealed the ALK:p.I1171N resistance mutation. A switch to brigatinib (180 mg/d) led to stable disease. Disease progression occurred after 15 mo of treatment and triggered another biopsy with a subsequent switch to lorlatinib (100 mg/d) in August 2020. Because of further tumor growth, yet another tissue biopsy was performed in March 2021, and palliative radiotherapy was offered because of infiltration of the spinal canal (thoracic vertebrae 6–9; 12 × 3 Gy). Chemotherapy with carboplatin and pemetrexed was initiated in June 2021 (Fig. 1A) but was poorly tolerated. Therefore, the patient requested cessation of further antineoplastic therapy and opted for best supportive care in July 2021.

Molecular Findings

At the end of 2016, an endobronchial biopsy was taken that tested positive for an EML4–ALK translocation (exon 6 NM_019063.5 to exon 20 NM_004304.5) with mutations in APC, commonly observed in ADCs, RANBP2, and IRS1 (Fig. 2A). The second biopsy taken in 2019, after relapse under treatment with crizotinib and alectinib, confirmed the initial finding of an EML4–ALK translocation and also tested positive for an ALK:p.I1171N resistance mutation besides showing additional mutations in POLE and TSC2.
Figure 1. The fishplot (A) represents the clonal evolution over time with the development of subclones marked with 1–6. Radiotherapy is indicated by arrows on top with fractionation and dosage (in gray). The arrows on bottom represent the time and length of treatment, and the white lines indicate the time points biopsies were taken. (B) Representative figures of the hematoxylin and eosin (H&E) and immunohistochemical (IHC) stains (either as single or double staining: TTF-1, CK5/6, p63, CK7, PD-L1) demonstrating morphology and protein expression of biopsies taken at the respective time points. Red indicates negativity for the respective marker; green indicates positivity.
Figure 2. (A) The variant allele frequencies (VAFs) of cluster 1–6 for each sample generated with SciClone. Genes defining the respective clusters are shown in the diagram. (B) The subclonal architecture for the four biopsies with probabilities from ClonEvol. (C) The reconstructed phylogenetic tree with the protein changing mutations defining clones in the branches. The founding clones are marked with an asterisk. (D) The genomic regional copy-number variants (CNVs) of the four biopsies are shown. The y-axis gives the fold change of the coverage compared to a panel of normals with 1 standing for copy-number-neutral. The black lines with the alternating orange and blue (color change for better visibility) data points represent the mean of called segments. The red highlighted positions indicate early CNV events common for all four samples before diverging.
The biopsies taken in 2020 and 2021 shared a common set of additional mutations along with \textit{MLH1}, \textit{KMT2D}, and \textit{NFE2L2}. The initial \textit{EML4}–\textit{ALK} translocation and most of the mutations of the initial biopsy were also present, but the 2020 and 2021 biopsies were lacking the resistance mutation detected in 2019.

We performed a phylogenetic reconstruction based on the genetic data generated in the course of clinical routine molecular diagnostics. First, we wanted to confirm that the tumor biopsies were of monoclonal origin. Second, we wanted to explore whether selection of a squamous component in the context of a mixed histology at baseline might have occurred.

To this end, a clonal clustering of single-nucleotide polymorphisms (SNPs) was performed using the R package SciClone, which resulted in six clusters (Fig. 2A,B). The phylogenetic tree (Fig. 2C) includes cluster 1 with alterations in \textit{EML4}–\textit{ALK}, \textit{APC}, \textit{RANBP2}, \textit{IRS1}, and \textit{ETS1}, which proves to be the common ancestor for all four tumor samples. Cluster 2 is an early subclone of the common ancestor with private mutations in \textit{ARID1A} and \textit{ZFHX3} detected in the 2016 ADC sample only. Cluster 3 represents a second ADC subclone with mutations specific to the 2019 biopsy; it contains the resistance mutation \textit{ALK}:p.I1171N as well as a \textit{TSC2} and a \textit{POLE} mutation. Cluster 4 is the common ancestor of both SqCC with \textit{MLH1}, \textit{NFE2L2}, and two \textit{KMT2D} mutations. The SqCCs sequenced in 2020 (cluster 5) and 2021 (cluster 6) both show a high count of additional private mutations acquired in a relatively short period of time. Both SqCCs showed a much higher tumor mutational burden (TMB) with 16.5 mut/mb in 2020 and 20.6 mut/mb in 2021, compared to the ADC samples from 2016 and 2019 with 2.4 mut/mb and 4.7 mut/mb, respectively.

For monoclonal origin confirmation, a copy-number variation (CNV) analysis of all four samples was performed (Fig. 2D). Results showed that all four samples share genomic alterations, marked in red, that can be attributed to an early common event before further divergence. Further, we found a loss of heterozygosity common in both SqCCs but not present in both ADCs for the entire p arm of Chromosome 3, 5q14.1-q23.2 and 7q22.2-q36.3.

**DISCUSSION**

At diagnosis, the patient exhibited the \textit{EML4}–\textit{ALK} fusion, an \textit{APC} and two \textit{IRS1} mutations and received crizotinib followed by alectinib. Although the \textit{EML4}–\textit{ALK} fusion is significantly more prominent in ADCs, Han et al. (2006) describe that down-regulation of \textit{IRS1} is mostly prevalent in SqCCs, indicating genetic features associated with a squamous phenotype in the initial tumor biopsy matching a truncating loss of function \textit{IRS1} mutation in all four samples. The development of a primary resistance mutation \textit{ALK}:p.I1171N under crizotinib and alectinib therapy is well-described in the published literature (Ou et al. 2015). But the ensuing phenotypic switch from an ADC to a SqCC as evident by morphology and IHC is a rare event, which has been described previously in a couple of case reports only (Table 1; Gong et al. 2019; Park et al. 2019; Kaiho et al. 2020; Zhang et al. 2021). Interestingly, the transdifferentiation occurred under targeted ALK–TKI-therapy in those five reported cases, in which the administration of alectinib at some point during treatment was a common aspect. Further the transformation from an ADC to a SqCC was suggested as alternate resistance mechanism by Park et al. (2019), who also found a \textit{MLH1} mutation after SqCC transformation, similar to the presented case here.

The mutations in cluster 4 (\textit{NFE2L2}, \textit{KMT2D}, \textit{MLH1}; Table 2; Supplemental Table S2) are the common feature that differentiates the two later SqCC samples from the two ADC samples. Hence, they might have a causative role in triggering this transformation, although we cannot pinpoint a specific mutation without further functional analysis. The prevalence of \textit{NFE2L2} mutations is significantly higher in SqCCs compared to ADCs (Campbell et al. 2016), and the amino acid change p.E82D is classified as likely oncogenic (OnkoKB). The
role of KMT2D as tumor suppressor and epigenetic regulator could also be an attributing factor given that the presence of truncating mutations in KMT2D is much more prevalent in SqCCs (6.4%) than in ADCs (0.7%) (Cerami et al. 2012). As mentioned above a MLH1 mutation has been reported previously in a similar case (Park et al. 2019). Although loss of function of MLH1 is associated with DNA mismatch repair deficiency, the increased TMB of both SqCC samples compared to the earlier ADCs seems plausible, as the MLH1 mutation had the highest allelic fraction within the SqCCs founding cluster 4, followed by a rapid accumulation of further mutations in the later biopsies. Similarly, Gong et al. (2019) described in their report an increased TMB in the transformed SqCC of 15.17 mutations/mb. These molecular changes provide a rationale to adapt therapy, for example, giving immune checkpoint inhibitors. But the patient declined another line of treatment.

Table 1. Overview of previous published ALK-positive adenocarcinoma (ADC) and adenosquamous adenocarcinoma (AD-SqCC) case studies showing similar possible histological transformations

| Study            | Oncogenic driver | Phenotype                              | ADC sample | SqCC sample | Treatment               |
|------------------|------------------|----------------------------------------|------------|-------------|-------------------------|
| Chaft et al. 2012| ALK rearrangement| Synchronous ADC and SqCC lesions indicating AD-SqCC | TTF1+; p63--; CK5/6-- | TTF1--; p63++; CK5/6+ | Crizotinib              |
| Dragnev et al. 2014| ALK rearrangement| AD-SqCC                                | TTF1+; p63-- | TTF1--; p63++; p40+ | na                      |
| Gong et al. 2019 | EML4–ALK fusion  | ADC to SqCC transformation              | TTF1+      | TTF1--; p40+  | Crizotinib; ceritinib; alectinib |
| Park et al. 2019 | ALK-rearrangement| ADC to SqCC transformation              | TTF1+      | p63+        | Crizotinib; alectinib   |
| Kaiho et al. 2020| ALK rearrangement| ADC to SqCC transformation              | TTF1+; p40--; Napsin A+ | TTF1--; Napsin A--; p40++; p63+ | Alectinib; ceritinib |
| Zhang et al. 2021| ALK rearrangement| ADC to SqCC transformation              | TTF1+; p63--; CK5/6--; CK7+ | TTF1--; p63++; CK5/6+; CK7-- | Crizotinib; alectinib; brigatinib; lorlatinib |
| This study      | EML4–ALK fusion  | ADC to SqCC transformation              | TTF1+; p63--; CK5/6--; CK7+ | TTF1--; p63--; CK5/6--; CK7+ | Crizotinib; alectinib; brigatinib; lorlatinib |

The second column gives the corresponding driver alteration detected in the respective tumor. Columns 4 and 5 show the results of immunohistochemical (IHC) staining for the ADC or AD-SqCCs sample compared to the SqCC sample. The last column summarizes the TKI treatments the patients have received.

Table 2. Variant table

| Gene    | Chromosome | HGVS DNA reference | HGVS protein reference | Variant type  | Predicted effect | dbSNP/ dbVar ID | ClinVar accession | Interpretation |
|---------|------------|--------------------|------------------------|---------------|------------------|-----------------|-------------------|---------------|
| MLH1    | Chr 3      | c.G109C            | p.E37Q                 | SNV           | Nonsynonymous SNV | rs63751012      | SCV002058107    | Likely pathogenic |
| KMT2D   | Chr 12     | c.4379dupC         | p.L1461Tfs*30          | Indel         | Frameshift insertion | na             | SCV002058108    | Likely pathogenic |
| KMT2D   | Chr 12     | c.1940delC         | p.P647Hfs*283          | Indel         | Frameshift deletion | rs770315135     | SCV002058109    | Pathogenic/ likely pathogenic |
| NFE2L2  | Chr 2      | c.A246T            | p.E82D                 | SNV           | Nonsynonymous SNV  | na             | SCV002058110    | Likely pathogenic |

List of mutations only present in both squamous cell carcinoma (SqCC) samples with possible contribution to the transdifferentiation process from adenocarcinoma (ADC) to SqCC. SNV: Single-nucleotide variation.
The losses of heterozygosity found in both SqCCs but not in ADCs show no major prevalence in SqCC corresponding to the TCGA data set. Other prevalent CNVs in SqCC, like the recurrent amplification of the q arm of Chromosome 3, were not eminent, indicating no involvement of CNVs in the transdifferentiation in the presented case.

Despite the well-known subtype of adenosquamous carcinoma (AD-SqCC) of the lung showing traits of ADC and SqCC differentiation, and reports of ALK-translocated AD-SqCC cases, which were initially misclassified as SqCC because of sampling bias (Table 1; Chaft et al. 2012; Dragnev et al. 2014), the case presented here strongly indicates a true phenotype change as a possible resistance mechanism to treatment. The result of the genetic evaluation and the (immuno)histological examination clearly separate the two ADC samples from the later SqCC samples. The minor amount of p63-positive tumor cells in the initial biopsy does not qualify to dismiss the diagnosis of an ADC in favor of an adenosquamous carcinoma. Furthermore, the specificity of p63 for SqCC was described by our group to be imperfect in a study of 1244 NSCLCs, with ~16% of ADC showing positivity for p63 (Kriegsmann et al. 2019). Rather, the subset of p63-positive cells could indicate a subclone that bears the potential to differentiate toward a SqCC phenotype. Even if AD-SqCC cannot be completely excluded, one would have to assume extreme sampling bias leading to two clear ADC followed by two clear SqCC phenotypes, which is very unlikely. However, the recognition of only one histological component at a time in the biopsy at least suggests that there is a strong plasticity in which phenotype and genotype are variable under therapeutic pressure.

Phenotypic plasticity was described to play several roles in cancer development (Gupta et al. 2019). De-differentiation is a well-known mechanism for cancer progression and metastases. Trans-differentiation as an acquired resistance is an emerging theme, which is not well-understood. The presented case demonstrates such a trans-differentiation from ADC to SqCC with acquired alternative resistance mechanism to TKI therapy.

Furthermore, the diagnostic aspect that the SqCC biopsies do not refer to a second carcinoma but to the known primary tumor that has evolved molecularly and possibly phenotypically is of clinical importance and was only detectable by molecular workup. This also implies that clonality analysis by comprehensive molecular analysis on biopsies, as many patients are not detected until stage IV and complete histological workup via resection material is not possible, could be far more important in routine diagnostics.

Possible limitations of our research include the lack of a sequenced germline sample for a clean removal of germline SNPs, as well as the lack of a control baseline for the CNV analysis, but germline analysis is not routinely conducted because of ethical, legal, and economical constrains as all testing was carried out within clinical routine molecular diagnostics. Furthermore, a higher resolution of CNV detection and phylogenetic reconstruction could have been achieved with a whole-genome sequencing (WGS) approach. To obtain deeper insights into the transformation process, complementary analysis including epigenetics and transcriptomics, for example, would be needed. Possible further research in this area could include manipulating genes from cluster 3 in EML4–ALK-positive cell lines to trigger a possible transformation.

**Conclusion**

We support the transformation from ADC to SqCC as a possible alternative resistance mechanism under ALK–TKI-therapy similar to that shown in previous studies (Gong et al. 2019; Kaiho et al. 2020; Park et al. 2019; Zhang et al. 2021). This case study highlights the benefit of comprehensive molecular testing and reveals the evolutionary connection of the lesions despite their histological differences. Finally, we identified candidate molecular alterations...
That could have been causative for this rare event; thus, this case study may give rise to further in-depth investigations.

METHODS

Immunohistochemical staining of thyroid transcription factor 1 (TTF-1), CK7, CK5/6, p63, and PD-L1 (see Supplemental Table S1 for antibody details) were conducted using an autostainer (BenchMark ULTRA, Ventana Medical Systems) according to the manufacturer’s instructions. PD-L1 positivity was defined as linear membranous PD-L1 staining in ≥1% of tumor cells.

DNA was extracted automatically from six 10-µm FFPE sections of each sample by applying a Maxwell 16 FFPE Tissue LEV DNA Purification Kit on a Maxwell 16 Research system (both Promega). DNA concentrations were determined with the Qubit HS DNA assay (Thermo Fisher Scientific) according to the manufacturers’ protocols.

A TruSight Oncology 500 panel (Illumina) was used for targeted DNA sequencing according to the manufacturers protocol and as previously described (Kazdal et al. 2019). Mutation and CNV calling was completed by GATK 4.2. Only “PASS” mutations not present in gnomAD with a frequency of >0.1% and an allelic fraction of >2% in at least one sample were used for phylogenetic reconstruction. Fusion detection was performed either using a custom-designed (Pfarr et al. 2016) targeted RNA-seq panel (AmpliSeq technology, Thermo Fisher Scientific) or by using the anchored multiplex polymerase chain reaction (PCR)-based FusionPlex Lung kit (Archer DX) according to the manufacturer’s protocol.

For clustering, phylogenetic reconstruction, and plotting we used the R packages SciClone (Miller et al. 2014), ClonEvol (Dang et al. 2017), and fishplot (Miller et al. 2016), respectively.

ADDITIONAL INFORMATION

Data Deposition and Access
Mutations in Table 2 have been deposited in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) with the accession numbers SCV002058107–SCV002058110.

Ethics Statement
The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Tissues were used in accordance with the ethics committee of Heidelberg University (S-145/2017).

Author Contributions
P.C., A.S., and D.K. conceptualized the report; M.B., P.C., M.A., M.Ki, and R.B. established the methodology; M.B. was responsible for the software; M.B. and D.K. performed the validation; M.B., A.S., and D.K. performed the formal analysis; M.Ki., P.C., and D.K. performed the investigation; P.S. and A.S. provided the resources; M.Kr., R.B., and D.K. curated the data; M.B., M.A., P.C., M.T., A.S., and D.K. wrote the original draft; H.W., C.P.H., F.H., S.F., R.S., M.Kr., S.P., and A.S. performed the writing review and edited; Visualization, M.B. and D.K. performed the visualization; A.S. and D.K. supervised; and P.S. and A.S. administered the project.
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