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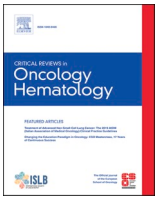

Tumor plasticity and therapeutic resistance in oncogene-addicted non-small cell lung cancer: from preclinical observations to clinical implications

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ABSTRACT

The identification of actionable targets in oncogene-addicted non-small cell lung cancer (NSCLC) has fueled biomarker-directed strategies, especially in advanced stage disease. Despite the undeniable success of molecular targeted therapies, duration of clinical response is relatively short-lived. While extraordinary efforts have defined the complexity of tumor architecture and clonal evolution at the genetic level, not equal interest has been given to the dynamic mechanisms of phenotypic adaptation engaged by cancer during treatment. At the clinical level, molecular targeted therapy of *EGFR*-mutant and *ALK*-rearranged tumors often results in epithelial-to-mesenchymal transition (EMT) and histological transformation of the original adenocarcinoma without the acquisition of additional genetic lesions, thus limiting subsequent therapeutic options and patient outcome. Here we provide an overview of the current understanding of the genetic and non-genetic molecular circuits governing this phenomenon, presenting current strategies and potentially innovative therapeutic approaches to interfere with lung cancer cell plasticity.

1. Introduction

Lung cancer is the leading cause of cancer-related death in many countries. The emergence of molecular-targeted therapy and immune checkpoint inhibitors (ICIs) has greatly improved the prognosis of patients with advanced stage cancer, especially those with non-small cell lung cancer (NSCLC). NSCLC patients harboring oncogenic mutations in driver genes can be successfully treated with specific targeted agents, mainly tyrosine kinase inhibitors (TKIs) (Tan and Tan, 2022). Although molecular-targeted therapies exhibit potent anti-cancer activity, almost all patients invariably relapse. At the molecular level, resistance mechanisms can be divided into two categories: 'on-target' and 'off-target' (Rotow and Bivona, 2017). The former mainly consist of secondary alterations in the kinase domain, that impair drug binding and efficacy, while off-target resistance is independent from the leading oncogene and includes the activation of alternative signaling pathways (bypass tracks). Moreover, phenotypic plasticity, namely the transient and reversible acquisition of a specific cell state without gain of any genetic alteration, is increasingly emerging as a key player in drug resistance in different tumor types, including lung cancer (Boumahdi and de Sauvage, 2020; Marine et al., 2020; Shen et al., 2020). Although many observations have arisen from preclinical studies, mechanisms of evasion to molecular-targeted therapy based on epithelial-to-mesenchymal transition (EMT), neuroendocrine (NE) and squamous cell carcinoma (SCC) transformation are well-documented also in the clinical setting. Remarkably, these phenotypic switches are generally unrelated to the driver genetic lesion. Overall, filling the gap between preclinical and translational studies could better define a novel roadmap to design

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innovative therapeutic strategies to overcome non-genetic mechanisms of drug resistance in lung cancer.

2. EMT-driven resistance

2.1. EGFR-mutated lung cancer

The epidermal growth factor receptor (EGFR) is a member of a tyrosine kinase family including human epidermal growth factor receptor 2 (HER2, also known as ERBB2), HER3 (ERBB3) and HER4 (ERBB4) (Yarden, 2001). EGFR activating mutations are localized within the ATP-binding site of the tyrosine kinase domain and result in the constitutive activation of the receptor in a ligand independent manner (Sharma et al., 2007). The most frequent targetable alterations of EGFR include exon 19 deletions and a missense exon 21 p.L858R point mutation, both conferring clinical responsiveness to anti-EGFR molecular targeted therapy (Lynch et al., 2004; Paez et al., 2004). Despite successful efficacy, acquired resistance to EGFR TKIs, including third generation compounds, remains a critical multifaceted challenge in medical oncology. Besides more common on/off-target alterations, several studies highlight phenotypic adaptation as a critical alternative mechanism of tumor escape (Fig. 1). Microarray analysis of erlotinib-resistant cells which developed EMT as a resistance mechanism revealed downregulation of *E-cadherin* and upregulation of mesenchymal markers (*vimentin* and *ZEB1*), without any acquisition of genetic alterations (Suda et al., 2011). Histone deacetylase (HDAC) inhibitor MS-275 moderately restored response to erlotinib in resistant cells, confirming the relevance of epigenetics rather than genetics in this specific context. In accordance with preclinical studies, EMT was subsequently observed in patients with *EGFR*-mutant NSCLC who acquired resistance against gefitinib or erlotinib without *EGFR T790M* mutation and/or *MET* amplification (Chung et al., 2011). At the molecular level, the AXL receptor tyrosine kinase correlates with EMT (Vouri and Hafizi, 2017) and its involvement in acquired resistance to erlotinib was first reported in 2012 in xenograft models of erlotinib-resistant cells (Zhang et al., 2012). AXL and vimentin were found overexpressed, while E-cadherin was downregulated and AXL inhibition by siRNA or small molecules restored sensitivity to erlotinib. Similar results were independently reported in another preclinical study, which identified AXL as a therapeutic target to overcome erlotinib resistance in NSCLC (Byers et al., 2013), suggesting also the possible association between AXL bypass signaling and EMT as critical drivers of EGFR-TKI resistance. At the post-transcriptional level, miR-127 negatively affected lung cancer vulnerability to gefitinib by promoting EMT and stem-like features. Mechanistically, miR-127 promoted NF-κB hyperactivation by blocking TNFAIP3 and NF-κB in turn sustained miR-127 expression trough a feedforward regulatory loop (Shi et al., 2017). At the transcriptional level, the switch between SOX2 and SOX9 was shown to control phenotypic plasticity and metastatic potential in lung cancer cells (Lin et al., 2016). Interestingly, Kuo et al. (2020) have recently demonstrated that *SOX2* silencing resulted in *vimentin* upregulation and *BCL2L11* down-modulation, a molecular asset that led to gefitinib tolerance. In contrast, ectopic expression of SOX2 in gefitinib-resistant cells decreased vimentin expression and restored sensitivity to gefitinib. Moreover, stimulation of TGF-β, a well characterized and potent EMT activator, downregulated *SOX2* and induced a mesenchymal phenotype responsible for resistance to both gefitinib and osimertinib (Kuo et al., 2020), a third-generation EGFR-TKI that has become the standard-of-care (SOC) in first-line treatment (Soria et al., 2018). Concordantly, a recent report investigating the biological basis of osimertinib resistance in patient-derived cells highlighted its association with EMT features (Yu et al., 2023). At the molecular level, resistant cells were characterized by the activation of TGF-β, YAP/TAZ and PI3K/AKT signaling pathways, as well as by the overexpression of ZEB1, YAP1 and AXL. Remarkably, the authors showed that osimertinib-resistant cells displayed an increased sensitivity to XAV939, a WNT-TNKS-β-catenin inhibitor, thus proposing its translational potential into the clinical setting.Thereby, the phenomenon of EMT seems not to be dependent on TKI generation but on a process of phenotypic adaptation to anti-EGFR therapy. Additionally, vimentin expression and Aurora kinase A (AURKA) activation were also observed after chronic treatment with third-generation EGFR TKIs (Shah et al., 2019). Accordingly, Aurora kinase inhibitors strongly potentiated the therapeutic benefit of anti-EGFR therapy through the upregulation of BIM

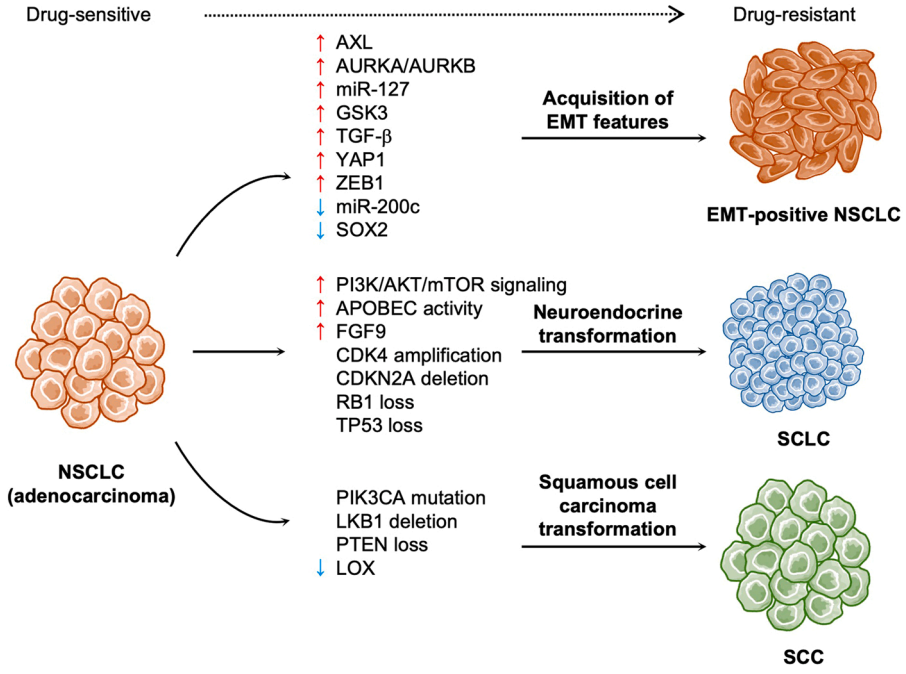

Fig. 1. Schematic representation of the main mechanisms of cell plasticity leading to phenotypic switch and drug resistance in molecular targeted therapy-treated non-small cell lung cancer (EMT: epithelial-to-mesenchymal transition; NSCLC: non-small cell lung cancer; SCLC: small-cell lung cancer; SCC: squamous cell carcinoma). Figure created with Servier Medical Art (smart.servier.com).

pro-apoptotic effector in both in vitro and in vivo preclinical models (Shah et al., 2019). Similarly, Tanaka et al. (2021) showed that ATR-CHK1-Aurora kinase B (AURKB), which regulates chromosomal alignment and segregation during mitosis, was activated in two cell lines exhibiting EMT-dependent osimertinib-resistance. Notably, AURKB inhibitor induced BIM-mediated mitotic cell death in both cell lines. Intriguingly, also cabozantinib, which inhibits multiple kinases including AXL, and glycogen synthase kinase 3 (GSK3) inhibitors were also able to overcome resistance to osimertinib conferred by EMT (Fukuda et al., 2020; Namba et al., 2019).

2.2. ALK-rearranged lung cancer

ALK rearrangement was first discovered by H. Mano's group (Soda et al., 2007). The most relevant chimeric variant in NSCLC is represented by the *EML4-ALK* translocation. However, many additional 5'end-chimeric partners capable to promote dimerization and constitutive activation of the ALK kinase domain have been reported (Ou et al., 2020). Several types of secondary mutations in the *ALK* gene, as well as *ALK* amplification, mediate resistance to ALK-TKIs. Additionally, activation of bypass tracks, such as EGFR, KRAS, KIT, MET, and IGF1R are involved in ALK-TKI resistance (Lin et al., 2017). Notably, also in this genetic setting, phenotypic plasticity plays a critical role in drug resistance. Fukuda et al. (2019) reported the involvement of EMT in acquired resistance to ALK-TKI in an *ALK* mutation-independent manner (Fig. 1). At the molecular level, in vitro and in vivo models revealed that EMT was related to miR-200c decrease and *ZEB1* upregulation independently from the type and generation of ALK inhibitor. Importantly, this program could be therapeutically overcome by pretreatment with the HDAC inhibitor quisinostat, which upregulated miR-200c and suppressed *ZEB1* expression. Furthermore, Gainor et al. (2016) identified the downregulation of E-cadherin and the upregulation of vimentin in 5 out 12 ceritinib-resistant biopsies, indicating the involvement of EMT in ceritinib resistance. However, 3 out of 5 patients concomitantly harbored secondary mutations in the *ALK* gene, suggesting that both acquired genetic resistance and phenotypic plasticity can coexist in the same tumor. Recondo et al. (2020) have investigated the mechanisms of lorlatinib resistance, proving EMT in two patient-derived cell lines but not in the primitive specimens, although ALK secondary mutations were preserved in both conditions. Several agents were assessed in combination with lorlatinib, leading to the identification of saracatinib and dasatinib (both SRC inhibitors) that, when administered with lorlatinib, exerted potent synergistic effects in the patient-derived cell lines. At the molecular level, saracatinib resulted in a down-modulation of actin stress fibers — a morphological change representative of EMT — partially supporting the hypothesis that the growth inhibitory effect of saracatinib might be related to the reversal of EMT. Finally, Tanimura et al. (2022) showed that EMT mediator ZEB1 is also responsible for HER3 activation in *ALK*-positive resilient cells. Concordantly, in vitro and in vivo models showed that afatinib, a pan HER family inhibitor, synergistically enhanced the antitumor effect of alectinib and brigatinib in *ALK*-rearranged cell lines with mesenchymal features.

Overall, these preclinical studies provide novel mechanistic insights into the process of EMT-driven drug resistance in both *EGFR*- and *ALK*-positive NSCLC (Fig. 1), suggesting novel therapeutic opportunities with potential translational relevance.

3. Neuroendocrine transformation

3.1. Clinical evidence

Although it is well established that SCLC transformation occurs in 2–15% of lung adenocarcinoma (ADC) cases under TKI treatment (Nosaki et al., 2016; Oser et al., 2015; Oxnard et al., 2011; Yu et al., 2013), only few pre-clinical models are available, and the vast majority of studies exploited clinical data and molecular annotation of primary specimens. Zakowski et al. (2006) initially documented a small cell lung cancer (SCLC) transformation of an *EGFR*-ADC after acquired resistance to erlotinib. Subsequent studies confirmed SCLC transformation after erlotinib failure in 14% of patients. Notably, molecular analysis revealed that the original mutational status of the *EGFR* gene was maintained, suggesting that SCLC transformation was not a secondary lung cancer (Sequist et al., 2011). Of note, phenotypic change into SCLC was considered specific for EGFR-TKI inhibitors since such transformation was not observed after chemotherapy or chemoradiotherapy. Importantly, no classical resistance mechanisms, such as *EGFR T790M* or *MET* amplification, were identified in transformed tumors, while a *PIK3CA* mutation emerged in one patient only upon SCLC transformation (Sequist et al., 2011). The clinical characteristics and outcomes of *EGFR*-mutated patients showing SCLC transformation at relapse to TKI therapy have been summarized in a systematic review by Xu et al. (2021). Briefly, the median time to SCLC transformation was 20.5 months and the original *EGFR* mutations were maintained in 86% of the patients. Unfortunately, none of the clinical characteristics were useful to find an association with SCLC transformation. Fujimoto et al. (2022) recently reported histological transformation after EGFR-TKI treatment using the largest population reported to date. Among the 2624 patients treated with EGFR-TKIs who underwent biopsy at progression, 2.2% developed histological transformation into high-grade neuroendocrine carcinoma (HGNEC), including 1.8% of pure SCLC. HGNEC transformation, including SCLC and large cell neuroendocrine carcinoma, was also observed upon treatment with first-, second- and next-generation ALK-TKIs (Caumont et al., 2016; Fares et al., 2020; Fujita et al., 2016; Miyamoto et al., 2016). A report by Lin et al. (2020) showed that the frequency of SCLC transformation after relapse to ALK-TKIs was 0.8% (2/263), with 0% (0/95) and 1.2% (2/168) in crizotinib- and next-generation TKI-resistant cases, respectively. Details about post-transformation therapies and outcomes of the SCLC-transformed cases discussed above, when available, are reported in Table 1. Additionally, Lin et al. (2020) reported an autopsied patient with *ROS1* fusion, who experienced SCLC transformation after failure of several ROS1 inhibitors, including crizotinib and lorlatinib. All lesions obtained from this autopsied patient histologically exhibited a SCLC phenotype while, at the genetic level, the *ROS1 G2032R* mutation responsible of crizotinib resistance disappeared. SCLC transformation was identified uniquely in this case among 65 ROS1 biopsied patients, revealing a frequency of 1.5%. Furthermore, SCLC transformation was reported in a patient with a *RET*-rearranged lung adenocarcinoma who relapsed to pralsetinib (Gazeu et al., 2023), a highly potent and selective oral RET inhibitor (Gainor et al., 2021).

3.2. Molecular insights

3.2.1. EGFR-mutated lung cancer

The genetics underlying NE transformation has been investigated by several studies (Fig. 1), although many aspects of the phenotypic switch remain unanswered (Quintanal-Villalonga et al., 2020). Indeed, *RB1* loss has been found in 100% of transformed SCLC (Niederst et al., 2015), but its deletion alone is not sufficient to explain ADC conversion into SCLC. Lee et al. (2017) investigated clonal evolution history of SCLC trans-differentiation in four patients, by longitudinally analyzing 9 lesions during EGFR-TKI therapy. All ADC exhibiting SCLC transformation harbored complete inactivation of *RB1* and *TP53* at diagnosis. Extending the analysis to 75 patients with EGFR-TKI resistance, IHC staining demonstrated that complete inactivation of RB1 and TP53 was more frequent in patients switching to SCLC (82.4% vs 3.4%), revealing a 43-fold greater risk of SCLC transformation in patients with *RB1* and *TP53* biallelic loss than in those without such inactivation (Lee et al., 2017). Furthermore, the hypermutation signature that characterizes the apolipoprotein B mRNA-editing enzyme, catalytic polypeptide (APOBEC) was frequently identified in the initial ADC branch that later transformed to SCLC, suggesting a potential functional role of APOBEC

Table 1
Selected reports of HGNEC transformation-mediated resistance against EGFR- and ALK-directed TKIs.

Reference	Sequist et al. (2011)	Xu et al. (2021)	Fujimoto et al. (2022)	Lin et al. (2020)
Number of patients with a re-biopsied sample	37	72	2624	245
Type of TKI before HGNEC transformation	First-gen EGFR-TKI (100%)	First-gen EGFR-TKI (85%) Second-gen EGFR-TKI (12%) Third-gen EGFR-TKI (3%)	First-gen EGFR-TKI (61%) Second-gen EGFR-TKI (32%) Third-gen EGFR-TKI (7%)	First-gen ALK-TKI (37%) Second-gen ALK-TKI (47%) Third-gen ALK-TKI (16%)
Frequency of HGNEC transformation	14%	NA	2.2%	0.8%
Frequency of SCLC transformation	14%	[a]	1.8%	0.8%
Median time to transformation (months)	NA	20.5	21.6[b]	NA
Median duration of primary TKI therapy (months)	14.1	18.5	NA	NA
First-line therapy after transformation	Platinum-etoposide	Cytotoxic CTx (66%) Cytotoxic CTx and/or RTx and/or EGFR-TKI (23%) Continued EGFR-TKI (5%) No anticancer therapy (6%)	Cytotoxic CTx (78%) ICI (10%) EGFR-TKI rechallenge (3%) No anticancer therapy (8%)	NA
Median PFS upon first-line therapy after transformation (months)	NA	4.0	4.1[c]	NA
Median OS upon first-line therapy after transformation (months)	NA	8.5	12.6[c]	NA

CTx: chemotherapy; HGNEC: high-grade neuroendocrine carcinoma; ICI: immune checkpoint inhibitor; NA: not available; NE: neuroendocrine; OS: overall survival; PFS: progression-free survival; RTx: radiotherapy; SCLC: small cell lung cancer; TKI: tyrosine kinase inhibitor.

[a] All patients included in the meta-analysis presented transformed SCLC

[b] Based on patients displaying transformation to HGNEC (including SCLC)

[c] Median PFS and OS of HGNEC

during SCLC trans-differentiation (Lee et al., 2017). A report by Offin et al. (2019) confirmed that *EGFR*-mutated patients with concomitant *TP53* and *RB1* mutations had a higher risk of SCLC trans-differentiation compared to those without such mutations (18% vs 0%). Additionally, it was shown that median time to transformation of patients with triple mutations in *EGFR*, *RB1* and *TP53* genes upon initial EGFR-TKI treatment was only 1.1 years. Irrespectively of transformation, the prognosis of triple mutants was worse than that of *EGFR/TP53* double mutants and *EGFR* single mutants (PFS: 9.5 m vs 12.3 m vs 36.6 m; OS: 29.1 m vs 40.8 m vs 56.4 m), suggesting that the population characterized by loss of oncosuppressor genes might be a special entity in the cohort of transformed SCLC, characterized by extremely poor prognosis. Notably, early preclinical data on mouse models had indeed showed that the conditional inactivation of Tp53 and Rb1 in lung neuroendocrine cells was sufficient to promote SCLC histology (Song et al., 2012), while their targeted disruption in alveolar type II cells was less frequently associated with transformation (Park et al., 2011; Sutherland et al., 2011). Recently, a pivotal role of fibroblast growth factor 9 (FGF9) in SCLC transformation and maintenance has been reported (Ishioka et al., 2021). Specifically, whole exome sequencing in one patient exhibiting trans-differentiation to SCLC upon TKI therapy confirmed the maintenance of the original *EGFR L858R* variant, a nonsense mutation of *RB1* and a missense mutation of *TP53*, while RNA-seq revealed upregulation of *FGF9*. FGF9 protein overexpression in post-EGFR-TKI samples was also confirmed by IHC (66.7%) in additional primary tumor samples. Interestingly, the expression level of *FGF9* positively correlated with that of *achaete-scute homologue 1* (*ASCL1*), one of the four transcriptional factors recently identified for the molecular classification of SCLC (Rudin et al., 2019). At the functional level, ectopic introduction of *FGF9* into *Tp53*- and *Rb1*-null murine ADC cells resulted in the acquisition of a SCLC-like phenotype. Of note, the pan-FGFR inhibitor AZD4547 suppressed growth of subcutaneous tumors derived from *FGF9*-overexpressing cells, suggesting FGF9 involvement in SCLC transformation through FGFR pathway activation. Intriguingly, in cells harboring *TP53* and *EGFR L858R/T790M* mutations, *RB1* knockout and *FGF9* overexpression under osimertinib chronic exposure resulted in SCLC transformation (Ishioka et al., 2021). Moreover, Quintanal-Villalonga et al. (2021) have recently comprehensively analyzed by whole exome sequencing, methylation analysis, RNA profiling and IHC a cohort of combined histology samples where ADC (T-LUAD) and SCLC (T-SCLC) coexisted. Almost all samples showed TP53 and RB1 protein expression loss, while recurrent loss of the 3p chromosome arm was frequently observed in T-LUAD only. In addition, alterations in the WNT, PI3K/AKT, Notch pathways and PRC2 complex were significantly enriched in T-SCLC compared with T-LUAD. This molecular signature was associated with the downregulation of RTK signaling, the inhibition of apoptosis and the suppression of antitumor immunity, thus suggesting that these alterations may have primed SCLC trans-differentiation. Finally, efficacy of a PI3K/AKT/mTOR inhibitor (samotolisib) was explored in a patient-derived xenograft of *EGFR*-mutant ADC transformed into SCLC upon EGFR-TKI treatment. The combination of samotolisib with osimertinib exhibited a more potent inhibitory effect compared to samotolisib alone. Intriguingly, osimertinib monotherapy completely depleted the ADC component, whereas the tumors treated with samotolisib alone were enriched in the ADC component, indicating that osimertinib and samotolisib could exert a distinct selective pressure on ADC and SCLC components, respectively. Thus, the AKT signaling axis may mediate transformed SCLC resistance against EGFR-TKI and the concomitant inhibition of EGFR and AKT signaling pathways may prevent *EGFR*-mutant ADC from transforming into SCLC (Quintanal-Villalonga et al., 2021). In conclusion, inactivation of *TP53* and *RB1* are essential in EGFR-positive tumors for SCLC transformation (1st and 2nd hits), but further alterations in gene expression, epigenetics and signaling pathways (3rd hit) are likely also necessary for the phenotypic switch.

3.2.2. *ALK-rearranged lung cancer*

The molecular bases of the NE transformation of *ALK*-positive adenocarcinomas have been investigated as well (Fig. 1). In *ALK*-rearranged ADC, Ishioka et al. induced SCLC trans-differentiation by ectopic expression of *FGF9* in *EML4-ALK* fusion-positive cells harboring *TP53*- and *RB1*-inactivating mutations. Although these data need further preclinical and clinical validation, they support a relevant role for FGF9 in the phenotypic switch, independently from the driver oncogene (Ishioka et al., 2021). According to Huang et al., amplification of cyclin-dependent kinase 4 (CDK4) and deletion of CDKN2A, which inhibits CDK4/6, both lacking in the primary tumor, were identified in the re-biopsied sample in a patient who developed SCLC transformation following treatment with three ALK inhibitors without changes in the RB1 or the TP53 mutational status (Huang et al., 2022). Signatures

associated with homologous recombination, mismatch repair and Notch pathways, as well as macrophages M2, were enriched in the SCLC sample. The authors suggest that CDK4 inhibition might be a potential therapeutic target to block SCLC transformation in ALK-positive patients, although further data are needed to support this approach. Furthermore, transformation into HGNEC, including SCLC, has been reported to confer resistance against ROS-1 inhibitors as well (Gendarme et al., 2022; Lin et al., 2020), confirming the impact of neuroendocrine transformation under TKI treatment, independently from the driver oncogene.

4. SCC transformation and other histologies

4.1. Clinical evidence

SCC transformation represents a rare mechanism of acquired resistance to TKIs and was first observed by two independent studies in 2015 (Hsieh et al., 2015; Kuiper et al., 2015). Fujimoto et al. (2022); Park et al. (2019a) showed that the frequency of SCC transformation from ADC during treatment with EGFR-TKIs was 1.1% and 0.4%, respectively, and no significant differences were observed in treatment outcomes between patients with SCC or HGNEC transformation. Possible involvement of SCC transformation in acquired resistance to ALK-TKI has been also reported (Park et al., 2019b), but its frequency and clinical significance have not yet been elucidated. Interestingly, also sarcomatoid transformation has been reported in *ALK*+ patients upon TKI treatment (Jiang et al., 2020; Kobayashi et al., 2013), supporting again the relevance of phenotypic adaptation as a mechanism of resistance to molecular targeted therapy.

Overall, analogously to EGFR- and ALK- driven NSCLC, SCC transformation might likely be observed in the future even in patients harboring alterations of other key oncogenes such as *ROS1*, *BRAF*, *MET*, *RET*, *NTRK* and *KRAS* treated with small molecules inhibitors.

4.2. Molecular insights

Regarding the potential mechanisms involved in SCC transformation during EGFR-TKI treatment (Fig. 1), Park et al. (2019a) investigated the genomic alterations in four patients with ADC who switched to SCC during EGFR-TKI treatment. Genetic dysregulation of the PI3K/AKT/mTOR pathway, such as *PTEN* loss, *PIK3CA* mutation and *LKB1* gene deletion were commonly found in all these patients after EGFR-TKI therapy. However, since very few patients have been genetically characterized in a longitudinal way so far, a clear molecular association of the factors involved in ADC transformation into SCC upon EGFR-TKI resistance has not been unequivocally determined (Park et al., 2019a). Notably, also murine Lkb1-deficient tumor models developed SCC, although in cooperation with Kras mutation (Hou et al., 2016; Ji et al., 2007), thus supporting the relevance of preclinical studies to dissect the mechanisms underlying SCC transition at the clinical level (Hou et al., 2016; Ji et al., 2007). Moreover, Han et al. showed, through in vivo lineage-tracing approaches in different Lkb1-deficient mouse models, that depletion of the *Lkb1* gene led to SCC transformation in a progressive manner, with a mixed adenosquamous cell carcinoma observed as an intermediate histological entity (Han et al., 2014). In this murine model, transformed SCC harbored specific pathway alterations compared to the original ADC, including cell-to-cell interactions and extracellular matrix (ECM) remodeling, such as a reduction of lysyl oxidase (LOX), which plays an important role in ECM assembly and upregulates *p63*, a critical SCC marker. *LOX* overexpression significantly inhibited ADC trans-differentiation, pointing to LOX-mediated ECM remodeling as a key player in the phenotypic switch. Additionally, both *Pten* and *Lkb1* loss in a mouse model resulted in a SCC that faithfully recapitulated the human disease not only in terms of genotype and phenotype but also at the immune-microenvironmental level (Xu et al., 2014).

Diverse plasticity routes exploited by oncogene-addicted NSCLC to escape molecular targeted therapy are schematically depicted in Fig. 1.

5. Targeting phenotypic plasticity

5.1. Current approaches

Multiple preclinical studies have shown potential therapeutic strategies to overcome phenotypic plasticity in response to anti-EGFR and anti-ALK TKI, including TGF-β, HDAC, AXL, Notch-1, GSK3, AURK, AKT, FGFR and SRC inhibitors. Unfortunately, no specific clinical trials have been designed to date to assess the efficacy of drug combinations in TKI-resistant *ALK*-positive NSCLC patients who developed EMT features, underlining the need of translational studies in this subgroup of patients. In contrast, several drugs targeting EMT have been investigated in clinical trials including also *EGFR*-driven NSCLC (Giaccone et al., 2015; Gray et al., 2015; Johnson et al., 2011; Mross et al., 2016; Neal et al., 2016; Reguart et al., 2014; Zhu et al., 2019) (Table 2). Notably, AURKA inhibitors are currently being evaluated in combination with osimertinib in an ongoing phase I clinical trial in *EGFR*-positive advanced NSCLC which developed resistance to osimertinib [NCT05017025; NCT04479306]. However, at present, none of the tested agents has been approved in the clinical practice.

Therapeutic strategies for SCLC-transformed patients after TKI failure include chemotherapy, TKI rechallenge, chemotherapy plus TKI and immunecheckpoint inhibitors; however, patients with transformed SCLC are generally treated with cytotoxic chemotherapy, which is the SOC for typical SCLC (Fujimoto et al., 2022; Sequist et al., 2011; Xu et al., 2021). According to the pooled analysis by Xu et al., median PFS and objective response rate (ORR) of chemotherapy after SCLC transformation in *EGFR*-mutated NSCLC patients were 4.0 months and 53%, respectively (Xu et al., 2021). Although TKI rechallenge is considered another therapeutic option upon SCLC transformation after TKI therapy, ORR of EGFR-TKI rechallenge for SCLC-transformed NSCLC was reported to be 15%, compared with an ORR of 77% using EGFR-TKIs before SCLC transformation (Fujimoto et al., 2022). On the other side, a case report on a TKI therapy-naïve, spontaneously transformed *EGFR*-mutant SCLC highlighted the temporary benefit of osimertinib treatment in this specific setting (Takuma et al., 2022). In addition to EGFR-TKI rechallenge, alectinib rechallenge was also described in a patient with an *ALK*--positive lung adenocarcinoma transformed into SCLC after relapse on alectinib, resulting in partial response and response duration of eight months, after resistance to chemotherapy (Yamagata et al., 2021). Combination of chemotherapy with EGFR-TKI (erlotinib, carboplatin and irinotecan) was effective also in a patient with SCLC transformation after EGFR-TKI failure (Lee et al., 2018). Considering that suppression of the anti-tumor immune response is one of the features of NE transformation (Quintanal-Villalonga et al., 2021), ICIs have been also explored in this context, showing very limited activity (2.1 months median PFS in patients with NE transformation upon relapse to EGFR-TKIs) (Fujimoto et al., 2022). Since *EGFR/RB1/TP53*-mutated patients exhibit high risk of SCLC transformation (Offin et al., 2019), a phase I study was designed and is currently underway to investigate first-line combination therapy of osimertinib and platinum-etoposide, a standard regimen for SCLC, for triple mutants (NCT03567642). On the other side, some studies are being designed to escalate treatment using chemotherapy plus TKIs in treatment-naïve patients with *EGFR*-mutant advanced NSCLC harboring baseline features which confer high risk of SCLC transformation upon single-agent TKI. However, these regimens are characterized by higher toxicity and narrow the treatment options at relapse.

5.2. Future potential strategies

Alternatively, we could start designing translational studies with a final perspective of specifically targeting cancer cell plasticity as well.

Table 2
Concluded clinical trials of drugs against EMT-associated pathways in lung cancer. The most advanced trials with supporting literature evidence are representatievly reported for each of the indicated targets.

Reference	Possible target	Agents	Phase of clinical trial	Tumor type	Result	ClinicalTrials.gov Identifier
Giaccone et al. 2015	TGF-β2	Belagenpumatucel-L versus placebo	III	Stage III/IV NSCLC (mainteinance therapy after platinum-based chemotherapy)	OS (primary endpoint) was not met	NCT00676507
Reguart et al. 2014	HDAC	Vorinostat in combination with erlotinib	I/II	NSCLC (second-line [after erlotinib relapse])	No meaningful activity was observed in combinational therapy of *EGFR*-mutated NSCLC patients after TKI progression	NCT00503971
Neal et al. 2016	AXL (also MET, RET, ROS1 and VEGF2)	Cabozantinib alone or in combination with erlotinib versus erlotinib alone	II	*EGFR* wild-type NSCLC (second- or third-line)	The study met its primary endpoint with PFS (PFS: 4.3 mo. for cabozantinib, versus 4.7 mo. for combination, versus 1.8 mo. for erlotinib)	NCT01708954
NA	Notch	RO4929097	II	NSCLC (second-line)	The study did not meet its primary endpoint of the response rate (PD: 100%)	NCT01193868
Gray et al. 2015	GSK3	LY2090314 in combination with CBDCA + PEM	I	Solid tumors including NSCLC (after SOC)	A safety profile of LY2090314 was established	NCT01286520
Johnson et al. 2011	SRC	Dasatinib	II	*EGFR*-mutant tumors (after erlotinib or gefitinib)	No activity observes in patients with *EGFR*-mutant lung adenocarcinoma and acquired resistance to erlotinib and gefitinib	NCT00570401
Mross et al. 2016	AURKB	BI 811283	I	Solid tumors including NSCLC (after SOC)	BI 811283 had a generally manageable safety profile	NCT00701324

AURKB: aurora kinase B; CBDCA: carboplatin; EMT: epithelial-to-mesenchymal transition; GSK3: glycogen synthetase kinase 3; HDAC: histone deacetylase; MET: MET proto-oncogene, receptor tyrosine kinase; NA: not available; NSCLC: non-small cell lung cancer; ORR: overall response rate; PD: progressive disease; PEM: pemetrexed; RET: RET proto-oncogene; ROS1: ROS proto-oncogene 1, receptor tyrosine kinase; SOC: standard of care; TGF-β2: transforming growth factor beta-2; TKI: tyrosine kinase inhibitor; VEGFR2: vascular endothelial growth factor receptor 2.

Based on the clear definition of the key drivers of NSCLC pathogenesis and the efficacy of available molecularly designed therapies in oncogene-addicted NSCLC, research studies should fill the gap between bench and bedside when dealing with tumor evolution beside the "hit the target(s)" approach, thus embracing complexity. As elegantly demonstrated by a barcode tracing approach (Bhang et al., 2015), even by using combination therapies to eradicate sparse pre-existing mutant clones, rare phenotypic traits can still emerge and predominate. This phenomenon likely recapitulates in vitro what clinicians observe in oncogene-addicted NSCLC patients who relapse by simply switching their histological features without acquiring any additional genetic variant. Thereby, the implementation of a robust pipeline would be desirable to dissect tumor cell plasticity at the molecular and functional level. An integrated multi-omic approach (genomic, transcriptomic and proteomic) at single cell resolution, including analysis of the tumor microenvironment, should be pursued in a longitudinal manner for each patient under TKI treatment. Furthermore, repeating biopsies at the time of response to first line TKIs and at relapse in advanced cases may shed new light on the same cell population in a far more complex system. This approach is now potentially feasible, as recently demonstrated in breast cancer patients under neoadjuvant chemotherapy (Kim et al., 2018) and in a small cohort of oncogene-addicted lung cancer patients (Maynard et al., 2020). Moreover, integration of these data with the analysis of circulating tumor DNA and circulating tumor cells is also mandatory to capture additional molecular and biological features responsible for histological transformation. Given the peculiar characteristic of oncogene-addicted tumor cells, a potentially successful approach in targeting cell plasticity could rely on adoptive immunotherapies. Although this approach is far from being applied in the clinical practice, a recent report showed that gene therapy targeting the KRAS G12D peptide in metastatic pancreatic cancer induced an objective regression with durable protective immune cells, making this strategy extremely appealing (Leidner et al., 2022). Furthermore, autologous tumor-infiltrating lymphocytes (TILs) therapy along with ICI targeting programmed death protein 1 (PD-1) showed feasibility in heavily treated advanced NSCLC, with some long-lasting response even in a patient with an *EGFR*-mutated tumor (Creelan et al., 2021). Additional therapeutic strategies may include cancer vaccines directed against potent immunogenic tumor-associated neo-antigens. In this setting, an ALK-directed DNA-based vaccine exerted a strong immune response in *Alk*-driven mouse models of NSCLC (Voena et al., 2015). Finally, personalized cancer vaccines alone or in combination with anti-EGFR TKIs are currently under investigation (NCT04397926; NCT04487093), opening novel perspectives not only for the treatment of oncogene-addicted NSCLC but also to design, in the next future, promising 'ad hoc' strategies for targeting with a different approach the dominant oncogene that is maintained in the transformed tumor.

6. Conclusions

Along with well-known genomic alterations, increasing knowledge has demonstrated the impact of non-genetic mechanisms in therapy evasion. Thus, targeting cell plasticity, although extremely challenging, represents a new opportunity to capture and earlier abrogate the emergence of full-blown drug-resistant populations. Despite recent advances, the vast majority of functional studies are exclusively pre-clinical. In the near future, it will be important to better clarify directly in patients the precise spatial and temporal dynamics engaged by tumor cell under treatment to switch their phenotype. In this context, the possibility to study the process of phenotypic adaptation in a longitudinal manner directly and specifically in the primary tumors, when feasible, or at least in the blood, could be crucial to capture all potential phenotypic traits and distinct lineage trajectories relevant for cellular transformation. Moreover, exploiting innovative pre-clinical models, such as organotypic cultures, with multi-omic technologies at single cell resolution may contribute to decipher the complexity of phenotypic adaptation, leading also to the identification of key vulnerabilities with a superior translational potential. Overall, the recent technological and conceptual advances in the field will improve the outcome of oncogene-addicted lung cancer patients and hopefully fuel the identification of a new roadmap for their cure.

CRediT authorship contribution statement

Gouji Toyokawa: Conceptualization, Investigation, Data curation, Writing – original draft. **Francesca Bersani:** Conceptualization,

Investigation, Data curation, Visualization, Writing – original draft. **Paolo Bironzo:** Conceptualization, Writing – review & editing. **Francesca Picca:** Data curation. **Fabrizio Tabbò:** Writing – review & editing. **Naoki Haratake:** Supervision. **Tomoyoshi Takenaka:** Supervision. **Takashi Seto:** Supervision. **Tomoharu Yoshizumi:** Supervision. **Silvia Novello:** Supervision. **Giorgio V. Scagliotti:** Conceptualization, Supervision, Funding acquisition. **Riccardo Taulli:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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