



# Tumor evolution in epidermal growth factor receptor mutated non-small cell lung cancer

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**Abstract:** As the incidence of cancer increases worldwide there is an unmet need to understand cancer evolution to improve patient outcomes. Our growing knowledge of cancer cells' clonal expansion, heterogeneity, adaptation, and relationships within the tumor immune compartment and with the tumor microenvironment has made clear that cancer is a disease that benefits from heterogeneity and evolution. This review outlines recent knowledge of non-small cell lung cancer (NSCLC) pathogenesis and tumor progression from an evolutionary standpoint, focused on the role of oncogenic driver mutations as epidermal growth factor receptor (*EGFR*). Understanding lung cancer evolution during tumor development, growth, and under treatment pressures is crucial to improve therapeutic interventions and patient outcomes.

**Keywords:** Epidermal growth factor receptor (EGFR); tumor evolution; non-small cell lung cancer (NSCLC)

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## Introduction

Cancer remains a major cause of mortality throughout the world despite extraordinary efforts over numerous decades to develop effective therapeutic interventions. Importantly, however, our understanding of the impact of genetic, epigenetic, and Darwinian adaptive evolutionary processes that concurrently impact cellular adaptation and the development of malignancies has dramatically improved (1). Hanahan and Weinberg initially proposed six “hallmarks of cancer” that enable tumor growth and metastatic dissemination which have been updated to include additional hallmarks as our understanding of cancer has improved (2). These characteristics can be acquired longitudinally in different sequences and by different mechanisms including mutations in DNA during replication, in repair machinery, and by exposure

to mutagens. Hence, malignancies are a heterogeneous group of aberrant cells with dysregulation of a set of core pathways (3). Non-small cell lung cancer (NSCLC) has become a model for understanding the intricacy of how complex genomic alterations, pathway dysregulations and external selective pressures drive tumorigenesis, evolution, and provide targets for therapeutic intervention. Here, we review the basic principles of cancer evolution as has been demonstrated in NSCLC as well as our understanding of how epidermal growth factor (*EGFR*) oncogene driven NSCLC evolves.

## The genetic basis of lung cancer

Early studies in human cancer genomes were limited to evaluating the sequential somatic mutations in specific oncogenes and tumor-suppressor genes (4). Studies

analyzing lung cancer development, identified loss of heterozygosity at chromosomal regions that encode tumor-suppressor genes at 3p21.3 (*RASSF1A*), 3p14.2 (*FHIT*), 9p21 (*p16*), and 17p13 (*p53*) as early events during the development of NSCLC (5). Further, the mutational landscape in the kinomes of multiple cancers, including lung, colon, and breast tumors among others, was analyzed and demonstrated that the majority of somatic mutations in cancer are “passenger” mutations that do not contribute to oncogenesis (6-12). When expanded to genome-wide sequencing, studies similarly demonstrated that tumors harbor thousands of genetic and epigenetic alterations that are not present in germline DNA of which only a very small fraction are oncogenic “driver genes” (13). These oncogenic driver genes function through a limited number of routes that regulate growth and survival pathways critical for oncogenesis (14,15). This is true for multiple cancer subtypes; however, lung cancer has become a paradigm of the power of utilizing targeted therapy to block these oncogenic pathways with the identification of *EGFR*, and other, activating mutations. The targetability of activating mutations in *EGFR* with tyrosine kinase inhibitor (TKI) was first demonstrated in 2004 (16-18). This was followed by the identification of rearrangements of the anaplastic lymphoma kinase (*ALK*) gene, which are uniquely sensitive to ALK TKIs and affect approximately 6% of NSCLC (19,20). In short succession, multiple other oncogenic drivers have been identified, most of which have targeted therapies that are commercially available or in clinical trial. These include more immediately targetable activating mutations such as *BRAF* (21,22), *ROS1* (23,24), neurotrophic receptor tyrosine kinase (*NTRK*) gene fusion (25,26), mesenchymal-epithelial transition factor (*MET*) amplification (27), human epidermal growth factor receptor 2 (*HER2/ERBB2*) (28-30), and translocations in *RET* (31-35); as well as those for which targeted therapy development has been more challenging such as Kirsten rat sarcoma virus oncogene homolog (*KRAS*) (36-38), and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) (39).

Previously, lung cancer had been thought to develop primarily due to carcinogen exposure from tobacco smoking (40). However, 25% of all lung cancer cases worldwide occur in never-smokers, representing 15% of lung cancers in men and 53% in women (40). Studies comparing the genomic landscape of NSCLC in never-smokers versus smokers have found several significant differences among these: (I) 10-fold higher mutation frequencies observed in smokers; (II) different mutation spectrum between smokers

(C:G → A:T) and never-smokers (C:G → T:A); and (III) distinctive sets of mutations identified in never-smokers (*EGFR* mutations and *ROS1* and *ALK* fusions) and smokers (*KRAS*, *TP53*, *BRAF*, *JAK2*, and *JAK3* and mismatch repair gene mutations) (41). Among these mutations, oncogenic mutations in the *EGFR* kinase domain occur early in the development of NSCLC in never-smokers, and *KRAS* mutations occur early in the development of smoking-related NSCLC (42,43). The discovery of these genomic alterations, pathway dysregulations, and external selective pressures that drive NSCLC tumorigenesis and evolution have become a model for understanding cancer development and the development of therapeutic targets.

### Oncogenic EGFR mutations in lung cancer

Based on two large comprehensive analyses of lung cancer, the Lung Cancer Mutation Consortium (LCMC) and the Cancer Genome Atlas (TCGA), it is known that almost 62% of patients with NSCLC harbor an oncogenic driver mutation (LCMC: 622/1,007; TCGA: 143/230) (44-46). Among those with oncogenic alterations, only approximately half have a therapeutically targetable lesion (44-46). These showed that *KRAS* is the most common oncogenic mutation occurring in 25–32% of patients (44-46). Somatic activating mutations in *EGFR* are the second most common driver mutation, occurring in 11–15% of all lung cancer patients (44-47). *EGFR* mutations occur with increased frequency in women, never smokers, and those of East Asian ethnicity, affecting 30–50% of patients with NSCLC in East Asia (48).

*EGFR* mutations can occur anywhere within the tyrosine kinase domain, however those associated with responses to TKI therapy are observed in exons 18–21. *In vitro* studies have identified 21 *EGFR* activating mutations, among 7,216 possible randomly mutated *EGFR* single-nucleotide variants (49). Clinically, the most common *EGFR* alterations are exon 19 deletions and the exon 21 L858R substitutions, which account for approximately 90% of *EGFR* mutations (50). Less common *EGFR* activating mutations include exon 20 insertions and exon 18-point mutations, which represent 1–17% and 3–4% of all *EGFR* mutations, respectively (50-58). These alterations lead to activation of the *EGFR* receptor and subsequent downstream activation of Ras/Raf/MAPK, PI3K-AKT-mTOR, and JAK-STAT signaling pathways, which induce cellular proliferation, apoptosis, angiogenesis, invasion, and metastasis (42,59-62). Patients with *EGFR*-mutant NSCLC often respond to first-

generation non-covalent EGFR TKI drugs such as gefitinib and erlotinib (16-18), but usually develop drug resistance within 9–12 months (63,64). Third generation TKIs such as osimertinib have a response rate of ~80% with an 18.9-month progression free survival (65). Importantly, the efficacy of EGFR TKIs varies among specific alterations, *EGFR* exon 19 deletions and *EGFR* L858R mutations in exon 21 show high rates of EGFR TKIs response versus *EGFR* exon 20 insertions which are associated with poor response to EGFR TKIs (53,66-68). Hence, comprehensive molecular genetic testing has become vital to the management of advanced NSCLC since the recognition of the predictive benefit of specific targeted agents varies among different oncogenic-driven tumors.

### Lung cancer heterogeneity and evolution

One of the critical components to understanding tumor evolution is to recognize the impact of tumor heterogeneity. Several large-scale data monitoring projects in different tumor subtypes have characterized and tracked intra-tumoral heterogeneity. A landmark study which demonstrated the importance of tumor heterogeneity was Gerlinger and colleagues' work with metastatic renal cell cancer in which they analyzed tumor tissue from primary and metastatic disease sites within the same patient (69). They found that 63% to 69% of all somatic mutations identified in one sample, were not found in geographically separate samples from the same patient, this demonstrated branched evolutionary tumor growth. In addition, and conversely, they specifically analyzed tumor-suppressor genes, which showed distinct inactivating mutations within a single tumor, revealing convergent phenotypic evolution (69). Their work also revealed that heterogeneity was present in all levels of analysis: genome, transcriptome, and proteome. In lung cancer, multiple groups have attempted to characterize heterogeneity and evolution. Among these, Imielinski and colleagues performed exome and genome sequencing of 183 lung adenocarcinoma tumors and identified novel oncogenic mutations in *U2AF1*, *RBM10* and *ARID1A* as well novel activating in-frame fusions of *EGFR* (70). Similarly, the Cancer Genome Atlas molecularly profiled 230 resected lung adenocarcinomas. They identified alterations in *NF1*, *MET*, *ERBB2* and *RIT1* in 13% of cases and showed these alterations were enriched in samples that otherwise lacked an activated oncogene (45). These findings suggested previously unrecognized driver roles for these genetic alterations, highlighting the diversity

among tumors and expanding the range of possible targetable alterations.

The Lung Tracking Cancer Evolution through Therapy (TRACERx) program is a translational longitudinal study aimed at understanding the mechanisms of cancer evolution by analyzing intratumoral heterogeneity and tracking its evolution from time of diagnosis to relapse. In their initial analysis with multi-region whole-exome sequencing of 100 early-stage NSCLC patients, they found that a median of 30% of somatic mutations and 48% of copy-number alterations are subclonal, highlighting that genomic-instability processes are ongoing during tumor development (71,72). Also, they observed substantial variation in intratumoral heterogeneity, reporting a wide range of number of subclonal mutations and percentage of the genome (0.06% to 81%) affected by subclonal copy-number alterations (72). When comparing histologic subtypes, squamous-cell carcinomas carried significantly more clonal mutations than adenocarcinomas ( $P=0.003$ ), potentially reflecting differences in smoking history (72). In adenocarcinomas, higher clonal and subclonal mutational burden was also observed in smokers. Tumor stage also correlated with the proportion of subclonal copy-number alterations in this group. Within this group, high copy-number heterogeneity was associated with an increased risk of recurrence or death ( $HR = 4.9$ ;  $P=4.4 \times 10^{-4}$ ), suggesting those patients represent a high-risk group which may require closer monitoring (72). The timeline of genetic alterations was also evaluated, leading to demonstration of early genome doubling and ongoing chromosomal instability as drivers of parallel evolution (72). Driver alterations as *EGFR*, *MET*, and *BRAF* were almost exclusively clonal and occurred early in evolution, compared to heterogeneous subclonal driver alterations in genes as *PIK3CA* and *NF1* which occurred later and were found in more than 75% of the tumors (72). These findings highlight the limitations of single diagnostic biopsies in accurately capturing intratumor heterogeneity and evolution.

In a follow-up analysis of Lung TRACERx, the authors explored allele-specific HLA loss and immune escape in the same cohort of patients, HLA loss was present in 40% of early-stage NSCLCs (73). HLA loss is an evolutionarily selected immune escape mechanism that is subject to strong microenvironmental selection pressures later in tumor evolution. It is associated with higher subclonal neoantigen burden, increased nonsynonymous mutations, APOBEC-mediated mutagenesis, upregulation of cytolytic activity, and PD-L1 positivity. Among those with stage IV disease,

HLA loss occurred preferentially at metastatic sites (73).

### Heterogeneity, evolution, and resistance of EGFR-mutant lung cancer

*EGFR*-mutant NSCLC has become a paradigm for understanding the complexity of how genetic alterations evolve over time and under treatment pressures leading to therapeutic resistance. Resistance to targeted therapies such as EGFR TKIs can develop by multiple mechanisms including: intrinsic resistance, adaptive resistance, and acquired resistance (74). Some tumors exhibit intrinsic resistance to treatment, such as those with *EGFR* exon 20 insertions, germline *BIM* deletion, and other baseline co-mutations (53,75,76). In contrast, adaptive resistance is observed when tumor cells undergo changes during therapy that permit their survival, an example is NF- $\kappa$ B signaling activation which is rapidly activated on initial EGFR TKI exposure to promote tumor cell survival and residual disease (77). Acquired mechanisms of resistance can arise from selection of clones with pre-existing genetic alterations within a heterogeneous tumor and from the acquisition of new alterations under the selective pressures imposed by therapy (74). Among these, *EGFR* T790M mutation has become a canonical *EGFR* mutation typically found in tumors with acquired resistance to first- and second-generation EGFR TKIs (78). The *EGFR* T790M mutation increases the affinity of EGFR for adenosine triphosphate (ATP), reducing the potency of ATP-competitive TKIs. This mechanism of resistance has been identified in around 50–70% of *EGFR*-mutant cases after treatment with a first- and second-generation TKIs, leading to the development of third-generation TKIs such as osimertinib (63,78–81). Similarly, the C797S mutation has emerged as a common resistance mechanism to osimertinib (82,83).

Prior efforts describing the complexity of lung cancer genetics were largely limited to resectable early-stage NSCLC leaving knowledge gaps in understanding the complexity of heterogeneity and evolution of oncogene-driven advanced-stage NSCLC, the role of co-occurring genetic alterations, and how these change with therapy, ultimately leading to the acquisition of drug resistance mechanisms. Among several recent studies to address these questions, Blakely and colleagues performed genomic analysis of 1,122 ctDNA samples from patients with advanced-stage *EGFR*-mutant NSCLC at different time points during treatment, compared to 1,008 *EGFR*-negative advanced-stage NSCLC samples (84). Canonical *EGFR*

mutations commonly co-occurred with subclonal oncogenic driver alterations in *PIK3CA*, *BRAF*, *MET*, *MYC*, *CDK6*, *AR*, and *CTNNB1* (84). To further understand the evolution of resistance in *EGFR*-mutant tumors they analyzed changes in ctDNA under EGFR TKI therapy. Those with progressive disease on first- and second-line TKI had an increased number of detectable somatic alterations which increased with each subsequent line of therapy, regardless of age, sex, or tobacco exposure (84). *EGFR* T790M and *EGFR* C797S cases were further analyzed, and demonstrated increased co-occurring genetic alterations and more frequent alterations in cell-cycle and WNT-pathway genes (84). Furthermore, cell-cycle-gene aberrations in *CDK4* or *CDK6* were associated with shorter progression-free survival (PFS) and overall survival (OS) to EGFR TKIs (84). In summary, they demonstrated that the number of somatic mutations increases during treatment and co-occurring genetic alterations may function as co-drivers of tumor progression and drug resistance leading to increased tumor genomic complexity and genetic diversity that facilitates further tumor evolution, adaptation, and resistance to therapy (84).

Piotrowska and colleagues analyzed tumor samples and circulating tumor DNA (ctDNA) in 12 *EGFR* T790M positive NSCLC patients who developed resistance to rociletinib (a 3rd generation EGFR TKI). By examining the heterogeneity of tumors and mechanisms of resistance, they observed small cell lung cancer transformation, acquired *EGFR* amplifications, and, half of tumors acquired T790-wild-type clones as mechanisms of rociletinib resistance (85). One biopsy showed coexisting T790-wild-type and T790M-positive clones prior to initiation of therapy. These and other findings demonstrated that tumors are composed of a mixture of cancer clones with different mechanisms of survival, some of which emerge under selective pressures (85). Separately, this group described the longitudinal molecular changes that occurred in a single tumor with an *EGFR* exon 19 deletion and *TP53* V173L metastatic lung adenocarcinoma from diagnosis and through therapy (86). This patient developed small cell transformation during erlotinib therapy, acquiring additional mutations in *PIK3CA*, *ERBB3*, and *FBXW7*. ctDNA profiling after progression on EGFR TKI revealed emergence of *EGFR* T790M for which osimertinib was initiated. Subsequent ctDNA analysis revealed that the *EGFR* T790M mutation became undetectable, but there were increases in *PIK3CA* and *EGFR* del19 mutations, and development of new *ERBB2*, *PIK3CA*, *c-MYC*, and

*FGFR1* amplifications (86). These cases highlighted the role of ctDNA in understanding the complexities of clonal evolution in acquired resistance, and illustrate how resistant subclones fluctuate in response to therapy.

The mechanisms of resistance to third-generation TKIs, such as nazartinib and osimertinib, among tumors harboring *EGFR* T790M were also described by Piotrowska and colleagues in a report analyzing the molecular profile of 2 patients with *EGFR* T790M treated with nazartinib (87). In one case, they observed the emergence of a new *BRAF* V600E subclone with concurrent decline of the *EGFR* T790M mutant allele fraction suggesting effective inhibition of the dominant *EGFR* T790M subclone and development of a new mechanism of resistance (87). At disease progression, they observed the emergence of a new *EGFR* T790M/C797S subclone in addition to the previously identified *BRAF* V600E-*EGFR* T790M-wild-type subclone (87). This highlights the heterogeneity of EGFR-dependent mechanisms of resistance and coexistence of multiple subclones which emerge at different points under treatment pressure. Similar findings were reported by Le and colleagues who analyzed a cohort of 118 patients with *EGFR* T790M mutations, 95% had progressed on at least one prior TKI and subsequently developed resistance to osimertinib (82). Molecular profiling showed that acquisition of *EGFR* C797S and L792 mutations were the most common EGFR-dependent resistance mechanisms and were observed exclusively in those who preserved an *EGFR* T790M mutation (82).

Other groups also evaluated how tumors evolve under the selective pressures of osimertinib. They identified known mechanisms of acquired resistance previously associated with resistance to first-generation EGFR TKIs such as small cell transformation, *MET* amplifications, *PIK3CA* mutations, and *BRAF* mutations, as well as novel mechanisms specific to third-generation EGFR TKIs such as new mutations, fusions, and/ or loss of *EGFR* T790M (83,88). Third-generation TKIs bind the *EGFR* C797 location, mutations in this locus within the *EGFR* gene, confer acquired resistance in 7–20% of patients (88,89-91). Further preclinical and clinical studies have identified that the allelic configuration (*cis* versus *trans*) of co-occurring *EGFR* C797S and *EGFR* T790M mutations may have therapeutic implications, with those in *trans* configuration having responses to combination of first- and third-generation EGFR TKIs (92,93). Other mechanisms of osimertinib resistance include *RET*, *FGFR3*, and *BRAF* fusions, as well as mutations in *KRAS* Q61K and *EGFR*

G796D and *MET* amplifications (83,94). As previously shown by Blakely and colleagues, alterations of cell-cycle genes had prognostic implications and were associated with shorter PFS. Ultimately, these studies illustrate the critical role of heterogeneity in cancer growth and resistance, as well as, the therapeutic challenges of the available targeted-therapies in controlling tumor growth while tumor cells undergo dynamic evolution under treatment pressure leading to the survival of selected oncogenic subclones that drive resistance.

As noted above, alternate resistance mechanisms include activation of complementary signaling pathways, concurrent alterations to other oncogenic genes (as described above), transformation to small cell histology (95), and epithelial to mesenchymal transition (EMT). An elegant example of the activation of alternative signaling pathways was demonstrated in recent work by Shah and colleagues who identified Aurora kinase A (AURKA) as a mediator of non-genetic acquired resistance to third-generation TKIs (96). Persistent EGFR inhibition leads to activation of AURKA by its co-activator TPX2, this activation is maintained in drug-tolerant cells and those with acquired resistance (96). AURKA mitigates drug-induced apoptosis and contributes to pathways associated with resistance to EGFR inhibition, including NF- $\kappa$ B, extracellular-signal-regulated kinase (ERK), and EMT (96). In addition, based on preclinical studies the combination of EGFR TKIs and Aurora kinase inhibitors suppresses this adaptive resistance mechanism, and enhance the initial response to EGFR inhibitor thus, forestall acquired resistance (96). Our evolving understanding of heterogeneity and evolution of resistance in advanced NSCLC highlights the importance of developing strategies to prevent and identify earlier mechanisms of resistance as well as clinical trial strategies that can overcome multiple concomitant resistance mechanisms.

### Epigenetics of NSCLC and EGFR resistance

Epigenetic dysregulation has been identified as a critical factor in tumorigenicity and heterogeneity, and understanding mechanisms of resistance (97-99). Commonly recognized epigenetic mechanisms that can promote or inhibit tumor cell growth include DNA methylation, histone or chromatin modifications, and dysregulation of miRNAs. In NSCLC, resistant cancer cells have been shown to develop after aberrant promoter methylation of *CDKN2A* (100), *MLH1* and *MSH2* (101), *APC* (102), *RARB* (103), and *MGMT* (104)

under therapy. Resistance acquired by drug exposure may be reversed after prolonged drug withdrawal or with epigenetic therapy, as histone deacetylase (HDAC) inhibitors and DNA methyltransferase inhibitors (DNMTi), which may re-sensitize NSCLC cells to targeted therapy or chemotherapy (99). HDAC inhibitors have been shown to upregulate tumor suppressor genes involved in apoptotic pathways, as *TRAIL* and *DR5*, and inhibit the expression of pro-survival genes as *BCL2* (105-110). In pre-clinical models and early clinical trials, HDAC inhibitors, such as panobinostat, in combination with EGFR TKIs have shown some signal for overcoming resistance to EGFR TKI resistance (111-116). Interestingly, HDAC inhibitors can also increase immune activation by upregulating MHC I and II expression (105). This has led to multiple trials assessing the safety of HDAC inhibitors in combination with immune checkpoint inhibitors [ClinicalTrials.gov identifier: NCT02437136 (117), NCT02954991 (118), NCT03233724 (119), NCT02638090 (120), NCT03590054 (121), NCT02635061 (122), NCT02805660 (123)].

The epithelial-to-mesenchymal transition (EMT) is another example of a complex adaptive, epigenetic process in which transcription factors such as TGF- $\beta$  induce the conversion of cells from epithelial to mesenchymal state, resulting in increased capacity for cell invasion, migration and drug resistance (124-129). In NSCLC, this is a well-explored mechanism of resistance to apoptotic signaling triggered by cisplatin and acquired resistance to EGFR, ALK and PI3K inhibitors (130-135). Resistant *EGFR*-mutant NSCLC cells have shown loss of epithelial cell junction proteins such as E-cadherin and TTF-1 and elevation in mesenchymal markers as vimentin, ZEB1, and CD44 (136-141). EMT and reduction in E-cadherin has been described in 20–25% of EGFR TKI resistant cases that lack secondary mutations (95,131,142-144).

miRNAs are another type of epigenetic alterations that can regulate EMT, response and resistance to chemotherapy, radiotherapy, and targeted therapies as EGFR TKIs (145-148). Among these, miR-21 has been shown to induce gefitinib resistance by suppressing *PTEN* and activating *ALK* and *ERK* (149,150). In contrast, upregulation of miR-133b has shown improved outcomes in NSCLC patients treated with erlotinib in the second- or third-line setting (151). Preclinical studies have suggested a therapeutic potential of miRNAs by synergistically sensitizing both *EGFR* wild-type and mutant NSCLC cells to TKIs (152,153). Few phase I clinical trials have aimed to evaluate miRNAs with varying degrees of tolerability (ClinicalTrials.gov Identifier:

NCT02369198; NCT01829971) (154-156). Ultimately, the role of epigenetically directed treatments in constraining NSCLC tumor evolution and development of resistance must be further studied to identify the best therapeutic strategies for clinical implementation.

### Immunotherapy and EGFR-mutant NSCLC treatment response and evolution

Immunotherapy (IO) has radically changed the treatment paradigm for patients with stage III and IV NSCLC, providing significant therapeutic benefit to many patients when compared with classical chemotherapy regimens (157-162). However, clinical studies in patients with *EGFR*-mutant tumors and other targetable oncogenic activating alterations have shown limited benefit with IO and lower response rates when compared to those without oncogenic activating alterations (163). For example, a meta-analysis assessing the role of IO in second line treatment included a subgroup of 186 *EGFR*-mutant patients with advanced NSCLC and showed no improvement in overall survival with single agent IO when compared chemotherapy, docetaxel (HR =1.05, 95% CI: 0.70–1.55) (164). Similar results were observed in TKI-naïve *EGFR*-mutant NSCLC patients, in which a phase II trial of single agent pembrolizumab was closed early due to futility (165). Notably, in the majority (70%) of these *EGFR*-mutant patients, tumor PD-L1 expression was high (PD-L1  $\geq$ 50%), suggesting that PD-L1 is not a predictive biomarker in patients with *EGFR* activating mutations (165).

Pre-clinical studies have suggested that high PD-L1 expression in this population is driven by the *EGFR* activating mutation and inhibition of *EGFR* activation by EGFR TKIs reduces PD-L1 expression (166,167). Based on these observations clinical studies aimed to assess the potential synergistic effects of combination of IO and EGFR TKIs in NSCLC therapy. However, multiple clinical trials demonstrated significant increase in grade  $\geq$ 3 toxicities with combination of IO and EGFR- or ALK-directed TKIs, as well as decreased efficacy when compared to TKI monotherapy (168-174).

Interestingly, a subgroup analysis of the IMPOWER 150 trial including patients with advanced *EGFR*- or *ALK*-altered NSCLC who had progressed on TKI therapy showed improved overall survival with combination of chemotherapy, bevacizumab (anti-VEGF), and atezolizumab (anti-PD-L1) when compared to chemotherapy and bevacizumab alone (HR =0.59; 95% CI: 0.37–0.94) (175).

This observation is provocative, given the lack of clinical benefit observed on prior trials assessing the role of IO as 1st line and 2nd line therapy of advanced-stage *EGFR*-mutant NSCLC (163,165). Further studies are warranted to identify the subgroup of patients who are most likely to benefit from this quadruple combination therapy.

There are multiple ongoing efforts to further define the population of NSCLC patients who benefit from IO, these studies aim to characterize the immune compartment of patient tumors and its interactions with the tumor microenvironment. In a study evaluating *EGFR*-mutant NSCLC patients who progressed during EGFR TKI therapy and who were T790M negative, PD-L1 expression  $\geq 1\%$  and high density of tumor infiltrating lymphocytes were associated with longer progression free survival with subsequent IO (176). Further studies, have identified that TMB is lower among those with *EGFR*, *ROS-1*, or *ALK* oncogene when compared to wild-type tumors, possibly explaining the lack of benefit observed with IO in oncogene-driven tumors (177-179). In addition, existing or *de novo* somatic alterations acquired during EGFR TKI exposure can improve response to IO, such as those impacting MHC functionality and neoantigen presentation, but have not been reported in the existing data (73,180).

Studies analyzing the interactions between tumors and the immune system aim to find factors and pathways that promote immune-escape and tumor growth, as well as identify alternate targets with potential clinical impact. An example of a gene with this potential is the Human Endogenous Retrovirus-H Long Terminal Repeat-Associating Protein 2 gene (*HHLA2*), which encodes for protein ligand HHLA2 found on the surface of monocytes and is a member of the B7 ligand family that demonstrates T-cell co-inhibitory properties (181). HHLA2 was found to be widely expressed in lung cancer and, importantly, it is highly expressed in *EGFR*-mutant NSCLC when compared to other lung cancer subtypes (182). Further efforts to identify the role of IO and novel checkpoint inhibitor targets in *EGFR*-mutant as well as another oncogene-driven NSCLC are needed.

## Conclusions

Significant discoveries on our understanding of cancer cell growth, progression, and acquisition of drug-resistance highlight the complexity of the genetic, metabolic, environmental, and evolutionary processes that concurrently shape lung cancer evolution. While these discoveries

have allowed for the development of targeted therapeutic interventions, they have also highlighted the existence of dynamic evolutionary changes leading to cellular adaptation, and activation of bypass signaling pathways that fuel cancer progression. In NSCLC, understanding of the clonality of *EGFR*-mutant tumors with co-occurring mutations impacts responses to treatments and how these evolve in response to different therapeutic agents is vital to guide treatment selection and their sequence. Multifaceted analysis of the changing molecular features of the pathways driving NSCLC growth at baseline and throughout the course of therapy is required to take into account tumor evolution and development of drug resistance. As additional therapeutic targets are identified and novel therapies are developed, reliable and accessible tools are needed to monitor and capture tumor heterogeneity and its evolution over time.

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