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Review

Resisting Resistance: Targeted Therapies in Lung Cancer

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Drug resistance inevitably limits the efficacy of all targeted therapies including tyrosine kinase inhibitors (TKIs). Understanding the biological underpinnings of TKI resistance is key to the successful development of future therapeutic strategies. Traditionally, mechanisms of TKI resistance have been viewed under a dichotomous lens. Tumor cells are TKI-sensitive or TKI-refractory, exhibit intrinsic or acquired resistance, and accumulate alterations within or outside the target to promote their survival. Such classifications facilitate our comprehension of an otherwise complex biology, but are likely an oversimplification. Recent studies underscore the multifaceted, genetically heterogeneous nature of TKI resistance, which evolves dynamically with changes in therapy. In this Review, we provide a broad framework for understanding the diverse mechanisms of resistance at play in oncogene-driven lung cancers.

Trends

TKIs represent a highly efficacious class of cancer therapeutics. However, drug resistance invariably limits the clinical activity of most TKIs.

Universal, well-established mechanisms of TKI resistance include secondary resistance mutations within the target kinase, activation of bypass signaling tracks, dysregulation of downstream effector proteins, and phenotypic transformation.

TKI resistance evolves dynamically under the selective pressure exerted by each targeted therapy. In some (but not all) cases, the evolution of TKI resistance can lead to significant tumor heterogeneity within a given cancer.

Drug-tolerant or 'persister' cells can survive initial TKI therapy and serve as a reservoir for drug-resistant clones.

Rational therapeutic strategies targeting mechanisms of TKI resistance are critical to improving clinical outcomes.

TKI Resistance: A Pervasive Challenge

In 2002, I.B. Weinstein defined the phenomenon of 'oncogene addiction,' whereby cancer cells become excessively dependent on a particular 'driver' alteration for their survival [1]. Cancers with these dependencies exhibit exquisite vulnerability to drugs that inhibit the drivers, so-called targeted therapies. The past decade has witnessed numerous successes in targeting specific molecular subsets of cancer across a spectrum of different human malignancies; most notably non-small-cell lung cancer (NSCLC). Somatic activating mutations in *EGFR* were the first driver alterations characterized in NSCLC, and are found in 10–15% of non-Asian patients [2]. These mutations confer sensitivity to small molecule TKIs of epidermal growth factor receptor (EGFR), resulting in high response rates and prolonged progression-free survival [3–8]. Rearrangements involving the *ALK* gene are identified in 3–7% of NSCLCs, and predict a comparable degree of clinical benefit from anaplastic lymphoma kinase (ALK)-TKIs [9]. Genotype-directed therapy is now the standard of care in advanced NSCLC and has led to improvements in overall survival [10,11].

The inevitable barrier that limits the effectiveness of TKI therapy and tempers enthusiasm is the issue of resistance – today's pervasive challenge for long-term disease control. Cancer is at its core a microcosm of evolution. Its survival is driven by genetic diversity and longitudinal accumulation of mutations, influenced by the selective pressures of TKI therapy. These rudimentary yet intricate principles underlie the refractory nature of TKI resistance, which is traditionally categorized as primary (intrinsic) or secondary (acquired). In primary resistance, patients lack any treatment response to targeted therapy. In secondary resistance, patients initially achieve some clinical benefit, followed by disease progression. With the discovery of each

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oncogenic driver and targeted inhibitor, a growing number and diversity of resistance mechanisms are being defined. In this Review, we highlight the major concepts that have emerged from studying mechanisms of TKI resistance in NSCLC.

Overview of Oncogenic Drivers in NSCLC

In addition to *EGFR* and *ALK*, several other oncogenic drivers have been discovered in NSCLC. In lung adenocarcinoma, the most common histological subtype of NSCLC, actionable drivers are present in over 20–25% of patients [10,11]. These alterations include *ROS1* rearrangements, *BRAF* mutations, *RET* rearrangements, *NTRK1* rearrangements, *MET* amplification and exon 14 skipping mutations, and *HER2* mutations (Figure 1) [10–17]. An in-depth discussion of each driver and its signaling pathways is beyond the scope of this Review. Kinase inhibitors targeting each of these oncogenes are either the standard of care or undergoing active development in the clinic, providing a fertile ground for investigations of drug resistance.

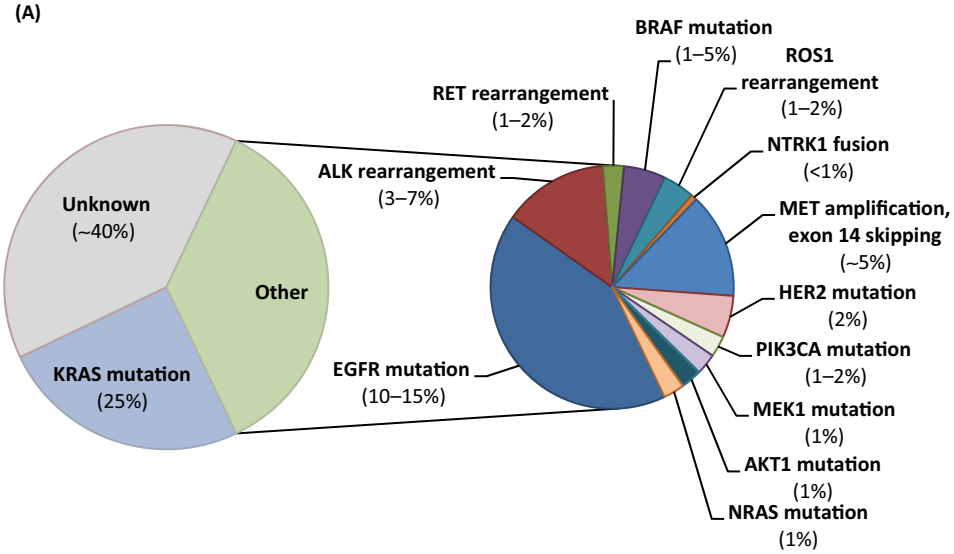
Primary Resistance

A review of the randomized trials using EGFR- or ALK-TKIs in the first-line setting for advanced *EGFR*- or *ALK*-positive NSCLC, respectively, suggests that primary resistance – when defined as 'progressive disease as the best response' – occurs in 4–10% of newly diagnosed patients [3–9]. Mechanisms underlying intrinsic TKI resistance are not fully elucidated but include non-sensitizing alterations within the target. For example, in-frame exon 20 insertions account for 4–10% of *EGFR* mutations [18–20]. These activate EGFR signaling *in vitro*, but do not confer sensitivity to first-generation EGFR-TKIs [18–21]. EGFR T790M, the gatekeeper mutation, is a well-described mechanism of acquired resistance to first-generation EGFR-TKIs (discussed below). In treatment-naïve patients with classic activating *EGFR* mutations, pre-existing EGFR T790M-mutant clones may promote intrinsic resistance at a certain threshold of allelic frequency [22,23]. The reported frequency of pre-existing T790M mutation has varied widely in the literature depending on the detection method (range, from <10% to 65%) [22–24].

Genetic alterations outside the target kinase can also contribute to decreased *de novo* sensitivity to TKIs. *MET* amplification has been reported in *EGFR*-mutant NSCLC prior to TKI exposure [25]. *BIM*, or *BCL2L11*, is a member of the BCL2 family that mediates apoptosis triggered by several TKIs. Recent studies showed that lower mRNA expression levels of *BIM* correlate with inferior efficacy of EGFR-TKIs [24,26]. Additionally, in Asian patients, a *BIM* polymorphism resulting in isoforms that lack the proapoptotic BH3 domain was associated with poor response to EGFR-TKIs [27]. A similar role for *BIM* has not yet been described in other oncogene-driven lung cancers. Another potential modulator of intrinsic NSCLC sensitivity to EGFR-TKIs is nuclear factor (NF)-κB. In *EGFR*-mutant lung cancer cells, RNAi-mediated knockdown of components of the NF-κB pathway enhanced erlotinib sensitivity, and similar effects were seen with pharmacological inhibition of NF-κB [28]. In a cohort of 52 patients with *EGFR*-mutant lung cancer treated with erlotinib, higher expression of the NF-κB inhibitor IκB was associated with increased TKI response and survival [28], suggesting that an approach combining EGFR inhibition with NF-κB pathway inhibition could potentially improve responses to EGFR-TKIs in the clinic.

Alterations in genes leading to the induction of epithelial-to-mesenchymal transition (EMT) have also been reported as a cause of primary TKI resistance [29,30]. For example, Park *et al*. demonstrated that overexpression of Cripto-1, an EGF-CFC family member, could confer erlotinib resistance in cell line and mouse xenograft models that was mediated by SRC activation [30]. In 85 NSCLC patients with *EGFR*-sensitizing mutations, higher Cripto-1 expression levels were associated with intrinsic resistance to EGFR-TKIs [30]. Further work is needed to validate these findings and establish whether Cripto-1 may be a major modulator of EGFR-TKI sensitivity in the clinic.

(A)



(B)

Inhibitors			
EGFR	**ALK**	**ROS**	**RET**
Erlotinib* Gefitinib* Afatinib* Osimertinib* Rociletinib EGF816 ASP8273 HM61713	Crizotinib* Ceritinib* Alectinib* Lorlatinib Brigatinib Ensartinib Entrectinib	Crizotinib* Ceritinib Lorlatinib Cabozantinib Foretinib Entrectinib DS-6051b	Alectinib Cabozantinib Vandetanib Lenvatinib Apatinib Ponatinib Sunitinib Dovitinib
MET	**TRK1**	**HER2**	**BRAF/MEK**
Crizotinib Tivantinib Cabozantinib Foretinib Volitinib Capmatinib MSC2156119J AMG337 AMG208	Entrectinib LOXO-101 DS-6051b	Afatinib Dacomitinib Neratinib Lapatinib Pyrotinib	Vemurafenib Dabrafenib Trametinib Selumetinib

Trends in Cancer

Figure 1. Oncogenic Drivers in Lung Adenocarcinoma. (A) The distribution of known oncogenic driver alterations in lung adenocarcinoma is shown, with estimated percentages for each driver. For an estimated 40% of lung adenocarcinomas, the underlying genetic alterations remain unknown. Approximately 25% of lung adenocarcinomas carry an activating *KRAS* mutation, for which targeted therapies are not yet available. (B) There are many tyrosine kinase inhibitors (TKIs) currently in use in the clinic or undergoing active development, which target the validated oncogenic drivers in non-small cell lung cancer (NSCLC). Examples are listed. Asterisks indicate TKIs which have been approved by the Food and Drug Administration for use in patients with NSCLC harboring the indicated genetic alterations.

Finally, false-positive genotyping may account for at least a few cases of apparent primary resistance. In the case of *ALK* rearrangement, the gold standard diagnostic assay has been a break-apart fluorescence *in situ* hybridization (FISH) assay. The most common *ALK* rearrangement, *EML4-ALK*, results from a small intrachromosomal inversion event, leading in some cases to a subtle splitting of the 5′ and 3′ signals [31]. This can lead to significant inter-observer variability and potential false-positive results [31]. Secondary confirmation of *ALK* positivity using immunohistochemistry or next-generation sequencing can thus be helpful.

Secondary Resistance

Extensive efforts in recent years have led to the elucidation of multiple mechanisms of acquired TKI resistance. Broadly speaking, the general categories of resistance mechanisms include secondary alterations within the target, activation of an alternative (i.e., bypass) signaling pathway or downstream effectors, and phenotypic transformation. Conceptually, all can be viewed as manifestations of evolution of cancer cells under the selective pressure of targeted therapies. Understanding each mechanism is paramount to developing therapeutic strategies to overcome, or even prevent, TKI resistance.

Mutations in the Target

Secondary somatic mutations within the target kinase enable its persistent activation despite the presence of the inhibitor. In general, these alterations hinder the ability of the kinase to bind the drug or alter the conformation of the kinase when noncontact residues are involved. The classic example is the gatekeeper ABL T315I mutation in Philadelphia chromosome-positive chronic myelogenous leukemia (CML). This mutation was first described in CML patients treated with imatinib, and has since been found to confer resistance to all approved ABL-TKIs prior to ponatinib [32]. T315I affects a conserved amino acid within the catalytic cleft that determines the relative accessibility of inhibitors to a hydrophobic pocket, resulting in steric interference with the binding of ABL-TKIs, but preserved kinase activity [32].

In *EGFR*-mutant NSCLC, the T790M gatekeeper mutation in exon 20 of *EGFR* was one of the earliest TKI-resistance mechanisms reported. It represents the dominant cause of resistance to erlotinib or gefitinib, seen in 50–60% of cases (Table 1) [33–35]. Interestingly, the T790M substitution appears to render resistance primarily by enhancing the kinase affinity for ATP

Table 1. Known Secondary Resistance Mutations in *EGFR*, *ALK*, *ROS1*, and *NTRK1*[a,c]

Mutation	*EGFR*	*ALK*	*ROS1*	*NTRK1*
Gatekeeper	T790M	L1196M	NR	NR
ATP-binding pocket	T854A	G1269A/S V1180L	NR	G667C[b]
Solvent front	NR	G1202R D1203N S1206C/Y	G2032R D2033N	G595R[b]
Covalent drug binding residue	C797S	NR	NR	NR
Other known mutations	L747S D761Y	G1123S I1151Tins L1152P/R C1156Y/T I1171T/N F1174C/L/V L1198F F1245C		

[a]Only those mutations clinically identified in patients are shown.
[b]This mutation has been identified in NTRK1-rearranged colorectal cancer patients treated with entrectinib.
[c]Abbreviations: NR, not reported.

rather than by causing steric hindrance [36]. Other non-T790M resistance mutations within EGFR are rarely observed in the clinic. These include T854A, D761Y, and L747S [34–39]. *In vitro*, the non-T790M mutations confer less potent resistance, and the mechanisms by which they confer resistance are not as well understood.

Recently, studies have provided insights into resistance mutations against third-generation EGFR inhibitors that are designed to target T790M, including osimertinib, rociletinib, and WZ4002. These compounds bind EGFR covalently through the cysteine residue 797. A novel EGFR C797S mutation, which alters the drug contact site, has now been observed in ~20% of patients who progress on osimertinib [40–43]. Notably, *in vitro* studies by Niederst *et al*. suggest that the allelic configuration of the C797S and T790M mutations may have an impact on the responsiveness of *EGFR*-mutant lung cancer cells to select groups of EGFR-TKIs [43]. Those cells with the C797S occurring in *trans* (on a different allele) with T790M were resistant to third-generation EGFR-TKIs, but retained sensitivity to combination therapy using a first-generation and third-generation TKI. In contrast, when the C797S occurred in *cis* (on the same allele) with T790M, the cells were resistant to all EGFR-TKIs [43]. Furthermore, if the cells acquired C797S in the absence of T790M (which has not been reported clinically but may be seen in the future if patients are treated with a third-generation EGFR inhibitor up front), they were resistant to third-generation EGFR-TKIs but responded to first-generation inhibitors [43]. These findings may have future clinical implications in guiding the sequencing of EGFR-TKIs.

For *ALK*, numerous kinase domain mutations have been reported in patients with acquired TKI resistance (Table 1). The ALK-L1196M gatekeeper mutation confers resistance through steric interference, and it occurs at a lower prevalence compared to EGFR-T790M [44–47]. G1269A is the second most common ALK resistance mutation that causes resistance by interfering with TKI binding. Mutations I1151Tins, L1152R, and C1156Y are located near the αC-helix outside the drug-binding region, and may cause TKI resistance by increasing catalytic activity [45–47]. Solvent front mutations G1202R and S1206Y alter residues in the solvent-exposed region of ALK. These mutations lower the drug binding affinity [45–47]. The G1202R mutation in particular is refractory to most ALK-TKIs including crizotinib, ceritinib, alectinib, and brigatinib but is overcome by the third-generation TKI lorlatinib [48–50].

Most recently, the ALK L1198F resistance mutation was discovered in a patient who relapsed first on crizotinib caused by an ALK C1156Y mutation and subsequently was treated with lorlatinib [51]. This mutation changes the very residue used to enhance the selectivity of lorlatinib for ALK over other tyrosine kinases, again demonstrating the inherent mutational capacity of cancer cells under selective pressures. Interestingly, with the addition of L1198F to C1156Y (and to other crizotinib-resistance mutations), lorlatinib-resistant cells could be resensitized to crizotinib [51]. This finding was demonstrated in cellular and biochemical assays, and also in the patient who re-responded to crizotinib after failing lorlatinib. This case highlights the clinical utility of multiple sequential ALK-TKIs and the importance of serial biopsies to guide their selection.

It is worth noting that there appears to be a much more diverse spectrum of resistance mutations identified in *ALK* compared to *EGFR*, where the EGFR T790M mutation is essentially the sole, clinically predominant resistance mechanism after failure of first- and second-generation EGFR-TKIs. This may be due in part to the presence of a pre-existing activating mutation in *EGFR*, which constrains the ability of the kinase to acquire additional mutations while preserving its function [47]. By contrast, multiple different secondary mutations in *ALK* have been reported in resistant patient specimens. As a whole, these mutations account for only about a third of crizotinib-resistant cases. Interestingly, a narrower and distinct spectrum of resistance mutations is seen with each next-generation ALK-TKI

Table 2. Mechanisms of Acquired Resistance in *EGFR*- and *ALK*-Positive NSCLC Treated with TKIs[a,b]

Category	Alteration	Estimated frequency (%)	Refs
Resistance to EGFR-TKI	**EGFR *target alteration***	***~60***	
	T790M	50–60 (for 1st-generation EGFR-TKI)	[33–35]
	D761Y, T854A, L747S	1-2	[34–39]
	C797S	~20 (for 3rd-generation EGFR-TKI)	[40–43]
	EGFR amplification	8–10	[33,39]
	Bypass signaling tracks	***~20***	
	MET amplification	5–22	[33,34,59]
	HGF overexpression	1 of 2 cases reported	[60]
	HER2 amplification	12	[33,34]
	FGFR3 activation	1 case reported	[63]
	BRAF mutations	1	[64]
	CRKL amplification	1 of 11 cases reported	[65]
	NF1 reduced expression	4 of 10 cases reported	[66]
	Phenotypic changes	***3–10***	
	Transformation to SCLC	3–10	[33–35]
	Unknown mechanism	***10–20***	
Resistance to ALK-TKI	**ALK *target alteration***	***~28–46***	
	Secondary mutations in *ALK*	22–36	[44–47]
	ALK amplification	7–18	[46,47]
	Bypass signaling tracks	***~40–50***	
	EGFR activation	Up to 44	[45–47]
	c-KIT amplification and SCF overexpression	15	[46]
	IGF-1R activation	4 of 5 cases	[62]
	MEK1 mutations	1 case reported	[63]
	PIK3CA mutations	1 case reported	[63]
	MET amplification	1 case reported (for alectinib)	[61]
	SRC activation	Unknown %	[63]
	Phenotypic changes	***<5***	
	Transformation to SCLC	< 5 (case reports)	[72–74]
	Unknown mechanism	***~15–30***	

[a]Only those mechanisms clinically identified in patients are shown.
[b]Abbreviations: CRKL, V-Crk avian sarcoma virus CT10 oncogene homolog-like; NF1, neurofibromin 1; MEK1, mitogen-activated protein kinase kinase; PIK3CA, phosphatidylinositol-4,5-bisphosphonate 3-kinase, catalytic subunit α.

(Gainor *et al.*, submitted). However, in this setting, secondary resistance mutations are seen in 50–60% of patients, similar to the frequency of EGFR T790M in EGFR-TKI resistance cases (Table 2) [46,47]. The lower prevalence of on-target alterations seen with crizotinib may reflect less potent target inhibition compared to next-generation ALK-TKIs, or EGFR-TKIs in *EGFR*-mutant NSCLC.

Secondary mutations in the target are now being reported in other oncogene-driven lung cancers as well. Solvent front mutations in *ROS1*, G2032R (analogous to ALK G1202R) and D2033N cause crizotinib resistance in *ROS1*-rearranged NSCLC (Table 1) [52,53]. The

ROS1 gatekeeper mutation L2026M confers resistance to crizotinib *in vitro* [54] but has not yet been observed clinically. For *NTRK1*, secondary resistance mutations against entrectinib were recently described in colorectal cancer, including the G595R solvent front mutation (analogous to ALK G1202R), and the G667C mutation (analogous to ALK G1269A and EGFR T854A) [55].

Target Amplification

Increased gene dosage through target amplification is a well-known cause of acquired TKI resistance. *EGFR* amplification has been identified in tumors resistant to EGFR-TKIs [33,39]. In one series of 37 patients with TKI-resistant *EGFR*-mutant NSCLC, three patients (8%) acquired *EGFR* amplification post-treatment [33]. All three patients, however, also acquired a *T790M* mutation with selective amplification of the *T790M* allele in two of these patients – suggesting that *EGFR* amplification may serve to enhance the resistance phenotype of the gatekeeper mutation [33]. Intriguingly, loss of the activating *EGFR* has also been reported in cases of EGFR-TKI resistance [56,57]. However, in these cases, there was concomitant reactivation of a downstream survival pathway [56] or histological transformation [57], which can abrogate dependence on the target oncogene for survival. Therefore, it is more likely that the loss of *EGFR* is a reflection of this state of target independence than a direct cause of acquired resistance.

Crizotinib resistance due to *ALK* amplification has similarly been observed in 7–18% of cases, and can occur alone or together with an *ALK* resistance mutation [46,47]. In one *in vitro* model, partial crizotinib resistance acquired through wild-type *EML4-ALK* amplification was then followed by a higher degree of resistance conferred by the emergence of ALK L1196M, suggesting a stepwise evolution of acquired resistance [58]. In all of these examples, tumor cells remain dependent on the target kinase.

Bypass Signaling Pathway Activation

Tumor cells orchestrate a network of signaling pathways that sustain survival. When a dominant pathway is inhibited by a TKI, diversion to parallel signaling pathways can allow cells to reactivate critical downstream effectors, allowing continued tumor survival and growth. As an example, *MET* amplification was one of the first described mechanisms of EGFR-TKI resistance in 2007 [59], and has since been identified in 5–10% of resistant patients [33,34]. *MET* amplification leads to phosphorylation of HER3 and reactivation of PI3K/AKT signaling, bypassing EGFR. Overexpression of the MET ligand, hepatocyte growth factor (HGF), similarly results in EGFR-TKI resistance [60].

Oncogenic drivers in NSCLC are essentially mutually exclusive, but can operate in a complementary manner in the resistant setting. *MET* amplification mediating EGFR resistance is one such example. In *ALK*-rearranged NSCLC, crizotinib resistance is mediated by EGFR activation in up to 44% of cases, although caused by upregulation of the receptor and/or overexpression of EGFR ligands rather than by genetic alterations of *EGFR* [45–47]. *MET* amplification has also been reported as a mechanism of resistance to the next-generation ALK inhibitor alectinib in *ALK*-rearranged NSCLC [61].

Other examples of bypass signaling which have been validated clinically in patient tumor samples include (Table 2): c-*KIT* amplification and ligand stem cell factor (SCF) overexpression, insulin-like growth factor-1 receptor (IGF-1R) activation, and SRC signaling upregulation in ALK-TKI resistance [46,62,63]; *HER2* amplification, fibroblast growth factor receptor 3 (FGFR3) activation, *BRAF* mutations, *CRKL* amplification, and *NF1* downregulation in EGFR-TKI resistance [33,34,63–66]; and EGFR activation in crizotinib-resistant *ROS1*-positive NSCLC [67]. Several additional resistance mechanisms have been proposed based upon preclinical studies but a description of these is beyond the scope of this Review. In all of these cases, the bypass

track reactivates a key downstream pathway in NSCLC, namely the PI3K/AKT/mTOR or RAF/MEK/ERK pathway [14].

Downstream Effector Activation

Tumor cells can also acquire alterations in downstream signaling effectors leading to drug resistance. A salient example occurs in *BRAF*-mutant melanomas, which develop resistance to BRAF inhibitor monotherapy through reactivation of the mitogen-activated protein kinase (MAPK) pathway [68]. MAPK reactivation has also been identified as a resistance mechanism to dabrafenib in a patient with *BRAF*-mutant NSCLC [69]. These preclinical findings provided the rationale for the development of combinatorial regimens of BRAF and MEK inhibitors in both melanoma and NSCLC harboring activating *BRAF* mutations.

MAPK activation has similarly been identified as a predominant resistance mechanism in both *EGFR*- and *ALK*-positive NSCLC after treatment with EGFR- and ALK-TKIs, respectively. This is of particular interest, as multiple combination regimens of EGFR-TKI or ALK-TKI with a MEK inhibitor have entered early phase testing based on preclinical findings. For example, in *EGFR*-mutant lung cancer, resistance to EGFR inhibitors can be acquired through reactivation of extracellular signal-regulated kinase (ERK)1/2. This resistance is overcome by treatment with MEK inhibitors such as trametinib [70]. Trickler *et al*. additionally showed that acquired resistance to EGFR-TKIs could be prevented by upfront combination therapy using an EGFR inhibitor plus MEK inhibitor in *EGFR*-mutant lung cancer cell line and mouse models [70].

Recent work using patient-derived cell lines identified the *MEK1* activating mutation, MAP2K1 K57N, as a driver of resistance to ALK inhibition in a case of *ALK*-rearranged NSCLC [63]. In this model, dual blockade of MEK and ALK was effective in overcoming resistance. Primary dependency of ALK on MEK as the dominant effector oncoprotein in *ALK*-rearranged lung adenocarcinomas may account for this finding, as proposed by Hrustanovic *et al*. [71]. In their *in vitro* assays, inhibition of MEK – but not PI3K/AKT or Janus kinase (JAK) – could recapitulate the growth suppression phenotype conferred by ALK inhibition. Conversely, constitutive activation of MEK could rescue the cells from ALK inhibition [71]. Notably, treatment of the *ALK*-positive NSCLC cells with both an ALK inhibitor (ceritinib or crizotinib) and MEK inhibitor (trametinib) suppressed the emergence of resistance in cell line and mouse xenograft models [71], providing a compelling rationale for the clinical evaluation of this combination approach.

Phenotypic and Histological Transformation

Cancers may acquire TKI resistance through phenotypic transformation. Change in histology from adenocarcinoma to small-cell lung cancer (SCLC) is observed in 3–10% of cases of resistance to EGFR inhibitors, including third-generation EGFR-TKIs (Table 2) [33–35]. Sequencing of *EGFR* from repeat biopsies has revealed that the activating mutation in *EGFR* seen in the original adenocarcinoma is retained in SCLC, suggesting a phenotypic evolution of tumor cells rather than a *de novo* SCLC [33–35]. Several case reports have now also been published of *ALK*-rearranged NSCLC patients with SCLC transformation after progression on crizotinib or alectinib [72–74].

How histological transformation mediates TKI resistance remains to be elucidated. In one study by Niederst *et al*., *RB* loss was detected in 100% of the 10 *EGFR*-mutant NSCLC patient tumor samples that had transformed to SCLC at the time of drug resistance (versus 11% of the 9 cases that retained NSCLC histology) [57]. Furthermore, RNA expression profiling and hierarchical clustering analysis showed the resistant, SCLC-transformed *EGFR*-mutant lung cancer cells to be more closely related to classical SCLC than other, TKI-resistant NSCLC cell lines [57]. Hence, this subset of TKI-resistant lung cancers may adopt the genetic (and perhaps epigenetic) features of classical SCLC, which is generally insensitive to EGFR-TKI therapy.

Another mediator of TKI resistance is phenotypic change of NSCLC via EMT, an evolutionarily conserved program of transdifferentiation and a driver of tumor invasiveness [75]. EMT was observed clinically in 5% of patients with EGFR-TKI resistance [33], and reported in cell line models of ALK-TKI resistance [76]. This change may involve the activation of AXL through augmented expression of *AXL* or its ligand *GAS6*, observed in 20% and 25% of EGFR-TKI-resistant tumors, respectively [77]. IGF-1R and SRC/FAK signaling have also been implicated in EMT in the context of TKI resistance [78,79].

Drug-Tolerant Persister Cells

Intrinsic versus acquired resistance has traditionally been perceived as dichotomous processes, but in actuality, the demarcation may be less distinct (Figure 2). In 2010, Sharma *et al*. proposed the notion of 'drug-tolerant persister cells' [80]. In NSCLC and other cancers, small subpopulations of cells (range, 0.3–5%) can survive the initial exposure to drugs by adopting a reversible,

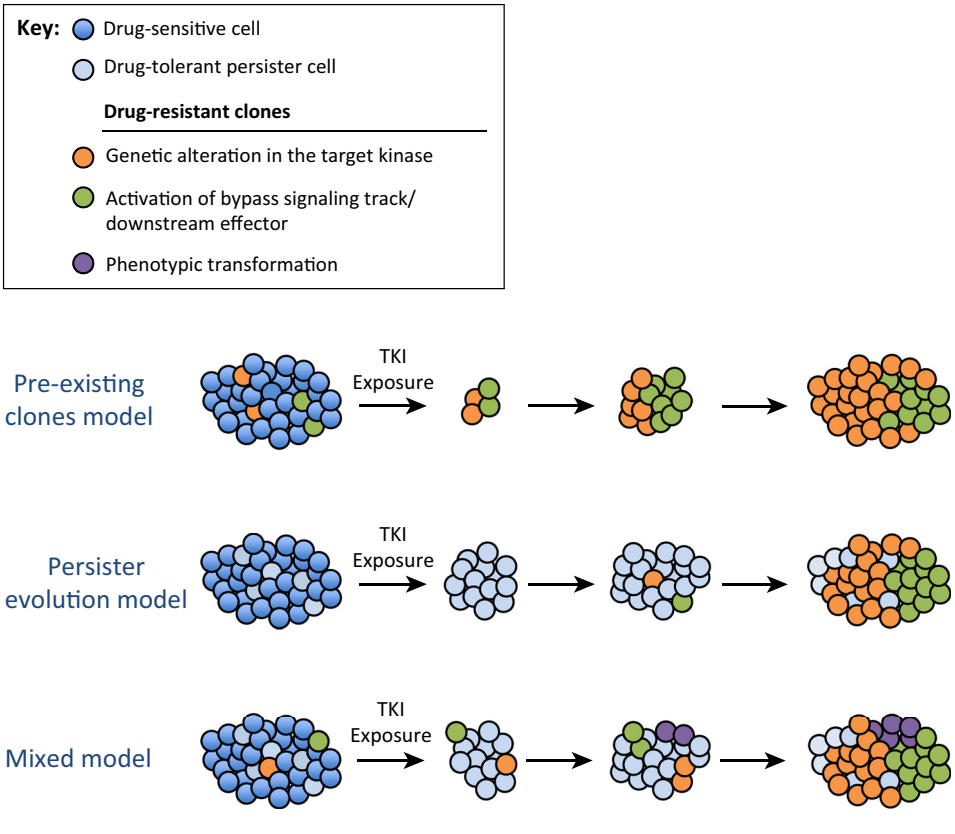


Figure 2. An Evolutionary Model of Drug Resistance in Cancer Cells. This figure depicts different models of the evolution of acquired resistance to targeted therapies in cancer cells. In the 'pre-existing clones model', a few tumor cells harbor a pre-existing resistance mechanism prior to the exposure to a tyrosine kinase inhibitor (TKI). These tumor cells with pre-established resistance mechanisms can survive and rapidly proliferate under the selective pressure of a TKI, becoming the predominant cell population. The tumor population as a whole will continue to be heterogeneous. Alternatively, in the 'persister evolution model', a small subpopulation of cells (represented in light blue) may adopt a relatively quiescent, reversible drug-tolerant persister state induced by the initial insult of the TKI exposure. This allows the survival of the persister cells and their subsequent propagation. The persister cells present a window of opportunity for the acquisition of resistance mechanisms, leading to the establishment of fully drug-resistant cells. In the 'mixed model', there are pre-existing drug-resistant clones as well as drug-tolerant persister cells in the TKI-naïve cell population, which evolve under the selective pressure of the TKI until fully drug-resistant tumor clones are established. The ratio of various drug-resistant clones will differ in each tumor and will dynamically change over time in concert with changes in therapy.

quiescent drug-tolerant state in response to the lethal stress. The emergence of drug tolerance is dynamic. Persister cells can emerge *de novo* from single cell-derived, drug-sensitive populations [80]. These persister cells subsequently propagate until a permanent, genetically based resistance mechanism is acquired [80]. Clinically, the majority of cancer patients with an objective tumor response to TKIs actually have incomplete (or partial) responses. While this residual disease burden may be secondary to multiple mechanisms (including pharmacokinetic issues that limit complete target inhibition), it could also be accounted for by the presence of persister cells, which ultimately lead to clinical relapse [81]. Therefore, understanding the mechanisms of persister cell survival may be critical in order to transform partial into complete responses and reduce the chance of resistance emerging.

Studies have demonstrated the importance of epigenetic programs in the emergence and survival of persister cells. Sharma *et al*. found that in some *EGFR*-mutant NSCLC cell lines, the development of EGFR-TKI tolerance requires activation of IGF-1R signaling. Increased IGF-1R phosphorylation was observed in persister cells, and treatment of the parental *EGFR*-mutant lung cancer cells with an EGFR inhibitor together with an IGF-1R inhibitor (AEW541) could suppress the emergence of persisters [80]. Interestingly, IGF-1R-mediated drug tolerance required the histone-demethylating activity of lysine-specific demethylase KDM5A, whose expression was upregulated in the persister cells [80]. KDM5A was previously shown to associate with histone deacetylases (HDACs), and its yeast ortholog Msc1 reduces histone H3K9/K14 acetylation. Indeed, the drug-tolerant *EGFR*-mutant lung cancer cells exhibited significantly reduced acetylation of histone H3K14, and treatment of these cells with an HDAC inhibitor (trichostatin A) resulted in cell death [80]. Altogether, these findings suggest a potential therapeutic role for HDAC inhibitors and/or IGF-1R inhibitors in suppressing the emergence of drug tolerance.

More recent work has identified additional mechanisms by which the persister cell state can be induced. For example, Blakely *et al*. reported that the treatment of *EGFR*-mutant lung cancer cells with an EGFR inhibitor could stimulate adaptive hyperactivation of NF-κB via ubiquitination events [82]. This NF-κB activation resulted in the upregulated expression of interleukin (IL)-6, activation of downstream JAK/STAT signaling pathway, and persister cell survival. In *EGFR*-mutant lung cancer cell line and murine xenograft models, pharmacological inhibition of NF-κB together with EGFR inhibition led to enhanced responsiveness to EGFR-TKI treatment, suppression of residual disease, and suppression of the emergence of acquired resistance, providing the rationale for clinical testing of this approach [82]. Additional work has shown that therapy-induced factors secreted into the tumor microenvironment (either by tumor or stromal cells) may also regulate the survival of persister cells [83]. Therefore, tumor cells likely utilize a variety of different mechanisms to survive the initial TKI exposure.

Once persisters survive the initial exposure to TKI, they may then go on to acquire a range of full-blown resistance mechanisms such as T790M in *EGFR* (Figure 2) [84,85]. Hata *et al*. studied the evolution of persister cells [85], and detected two distinct populations of EGFR-TKI-resistant tumor cells in *EGFR*-mutant NSCLC cell lines: 'early-resistant', pre-existing EGFR-T790M-mutant cells; and 'late-resistant', *de novo* EGFR-T790M-mutant cells. These late-resistant cells shared the molecular hallmarks of drug-tolerant persister cells [85], suggesting that persister cells likely served as the reservoir for subsequent T790M mutant resistant clones. Interestingly, compared to the pre-existing EGFR-T790M-mutant cells, the late-resistant cells exhibited diminished apoptotic response to treatment with a third-generation EGFR-TKI, WZ4002 [85]. The combination of WZ4002 with a BCL2-inhibitor, navitoclax, was able to induce enhanced apoptosis and tumor regression [85]. Based on these results, a clinical trial is underway examining the efficacy of osimertinib combined with navitoclax in patients with *EGFR*-mutant NSCLC after disease progression on first-line EGFR-TKIs.

Heterogeneity and Polyclonal Resistance

A binary view of resistance mechanisms fails in more ways than one. To this day, a resistant tumor is often simply labeled as either harboring one particular resistance mechanism or not. However, genetic heterogeneity is a defining feature of cancer and impacts TKI resistance, imbuing yet another layer of complexity.

Studies have demonstrated that in a given TKI-resistant tumor biopsy specimen, or in separate tumor sites within an individual patient, two or more discrete resistance mechanisms can be identified. Examples of polyclonal resistance in *EGFR*-mutant NSCLC include the co-occurrence of T790M with *MET* amplification [33–35,59], *EGFR* amplification [33], *HER2* amplification [34], or SCLC transformation [33,34]; and SCLC transformation with *MET* amplification [34] or *PIK3CA* mutation [33]. In *ALK*-rearranged lung cancer, concurrent ALK G1202R mutation and *c-KIT* amplification [46], G1269A and *ALK* amplification [47], and *MEK* mutation and *PIK3CA* mutation [63] have been reported. Clinically, though, durable disease control is often achieved by targeting the predominant mechanism of resistance despite this heterogeneity. One example can be found in T790M-positive, *EGFR*-mutant NSCLC, where despite the known heterogeneity (see below for further discussion), third-generation EGFR-TKIs appear to be typically uniformly effective [86,87].

In the clinic, heterogeneity in TKI resistance is also manifested temporally as patients undergo changes in therapy. 'Disease flares' occur in up to 25% of lung cancer patients who develop TKI resistance, usually within days of stopping the drug [88,89]. In parallel, 're-treatment responses' are seen: tumors presumed to be TKI-resistant reacquire sensitivity after a 'drug holiday' [88,89]. These phenomena represent clinical corollaries of the longitudinal evolution of heterogeneous tumors in concert with changes in therapy. Tumors with acquired resistance contain a mixed population of cells within which a small subset remains TKI-sensitive and proliferates rapidly upon the removal of the TKI, explaining the phenomenon of disease flare. During a drug holiday, repopulation of the cancer by TKI-sensitive cells leads to potentiation of responses upon TKI rechallenge [90].

More recently, Piotrowska *et al*. reported that T790-wild-type *EGFR* clones emerge after the T790M-positive *EGFR*-mutant lung cancers acquire resistance to rociletinib [87]. Single cell cloning of a T790M-'positive' pre-rociletinib specimen revealed both T790M-positive and -wild-type cells, suggesting that the apparent 'loss' of T790M at the time of resistance was actually due to the selection of these pre-existing T790-wild-type cells [87]. Similarly, Thress *et al*. found that some of the T790M-positive *EGFR*-mutant lung cancer cells treated with osimertinib could acquire resistance by 'losing' the T790M mutation [40]. This dynamic, polyclonal nature of resistance highlights the need for serial repeat biopsies to delineate key steps in the longitudinal evolution of resistance. As biopsies typically involve a single site and may not always be feasible, the development of noninvasive methods such as circulating tumor cell or cell free DNA analyses will be critical for evaluating the heterogeneity of resistance mechanisms.

Strategies Targeting TKI Resistance

Ongoing studies of TKI resistance mechanisms have shed light on the profound complexities of tumor biology and have guided the design of new therapeutic strategies to combat drug resistance. A detailed discussion of these strategies is beyond the scope of this Review; further information is available elsewhere [2,91]. Here, we close with a brief overview of the use of next-generation TKIs and TKI-based combinatorial regimens.

Secondary resistance alterations within the kinase have spurred the development of newer-generation TKIs. In *EGFR*-mutant NSCLC, several third-generation TKIs have been developed to

target T790M, including rociletinib, osimertinib, EGF816, ASP8273, and HM61713. Osimertinib is associated with a response rate of 61% and a median progression-free survival (PFS) of 9.6 months among patients with T790M-positive NSCLC who progressed on prior EGFR-TKIs [86]. The estimated duration of response is 6 months or longer in 88% of these patients [86]. Osimertinib is now FDA-approved for use in this patient population. Studies are underway to evaluate the efficacy of osimertinib in the front-line setting.

In *ALK*-rearranged NSCLC, the second-generation ALK inhibitors ceritinib and alectinib are currently FDA-approved for patients previously treated with crizotinib. In the ASCEND-1 trial, ceritinib showed a response rate of 56%, median duration of response of 8.3 months, and median PFS of 6.9 months in ALK inhibitor-pretreated, advanced *ALK*-rearranged NSCLC [92]. In ALK inhibitor-naïve patients, the response rate was 72%, with a median duration of response of 17 months and median PFS of 18.4 months [92]. In two phase II studies, alectinib demonstrated a ~50% response rate, median duration of response of 11.2–13.5 months, and estimated median PFS of 8.1–8.9 months in patients with advanced *ALK*-rearranged NSCLC who had progressed after crizotinib [93,94]. Clinical trials are ongoing to evaluate the efficacy of both ceritinib and alectinib in the first-line setting. Under active investigation are also a number of other next-generation ALK-TKIs, including lorlatinib, brigatinib, ensartinib, and entrectinib. Lorlatinib in particular has demonstrated clinical activity in patients who have failed multiple ALK-TKIs. Notably, each ALK-TKI is structurally distinct with varying degrees of activity against the different *ALK* resistance mutations *in vitro* and in the clinic [95]. These unique activity profiles may form the basis for selecting and sequencing next-generation ALK-TKIs.

The importance of bypass tracks and downstream effectors in mediating TKI resistance suggests that combinatorial approaches may be required. Indeed, numerous clinical trials are underway to evaluate the efficacy and tolerability of different drug combinations to overcome resistance. These include the inhibition of EGFR together with MET, HGF, mTOR, PI3K, MEK, BCL2, AXL/FLT3, SRC, IGF, JAK, or heat shock protein (HSP)90. In ALK-TKI resistant disease, co-inhibition of ALK with mTOR, CDK4/6, vascular endothelial growth factor (VEGF), MEK, or HSP90 is being studied. Several early-phase studies are also assessing the feasibility of coupling TKIs with immunotherapies, although this is based on limited preclinical data. The number of possible combination regimens is vast. The bottleneck in many cases will be the side effects that emerge when drugs are given in combination [2,91]. Use of alternative dosing schedules such as pulsed or intermittent dosing may help in certain cases. In parallel, greater rigor is warranted in the design of early studies to ensure the integration of patients' genotypes and resistance mechanisms in the rational design of combination strategies.

Concluding Remarks

Fourteen years have now passed since I.B. Weinstein's initial analogy of oncogene addictions to Achilles' heel [1]. Each year, novel tumor vulnerabilities are being discovered, and new therapeutic strategies are moving from bench to bedside. Amidst these advances, understanding TKI resistance mechanisms has become an urgent imperative. Many examples provided in this Review focus on our collective experience with *EGFR*- or *ALK*-positive NSCLC. However, TKI resistance is pervasive, and themes presented herein are likely universal. On the whole, the evolution of drug resistance is complex and dynamic – in many ways reminiscent of the multi-headed Hydra in Greek mythology [91].

Many questions on TKI resistance remain unanswered (see Outstanding Questions). Over the next decade and beyond, our ability to combat resistance will require collaborative efforts among bench, translational, and clinical researchers. More studies on the evolutionary dynamics of resistance are critical. Efforts will need to focus on the development of noninvasive diagnostic

Outstanding Questions

What molecular mechanisms underlie primary or intrinsic resistance to TKI therapy? On the opposite end of the spectrum, a small percentage of patients demonstrate prolonged responses to TKIs lasting many years. What are the molecular underpinnings of exceptional responses?

In some cases of resistance, cancers develop recurrent on-target gene alterations and remain oncogene-addicted. In other cases of resistance, cancers activate alternative signaling pathways and quickly lose their oncogene addiction. What is the biological basis for cancers evolving on-target versus off-target resistance mechanisms?

How do histological (i.e., SCLC transformation) and phenotypic (i.e., EMT) changes drive TKI resistance, and will these changes predominate as we develop increasingly effective targeted therapy approaches?

What are the key pathways enabling survival of drug-tolerant persister cells? What is the balance between rare pre-existing resistant cells and drug-tolerant persister cells, and how can therapeutic strategies be developed to target both populations?

Tumor heterogeneity has been widely described in resistant cancers, yet in many cases, one resistance mechanism predominates (e.g., EGFR-T790M). What are the best methods to assess tumor heterogeneity and to evaluate its clinical relevance?

What are the best upfront combination regimens to use to prevent acquired resistance?

tools to capture the full breadth of the evolving resistance mechanisms in patients. Thus far, our therapeutic strategies have focused on overcoming TKI resistance that is already established. It is likely that greater impact will be derived from novel therapeutic strategies that prevent the emergence of resistance upfront, target the drug-tolerant persister cells, and/or integrate targeted therapies with other treatment modalities such as radiation or immunotherapy. These approaches hold the promise of truly transforming cancer treatment and prolonging patients' lives.

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