Review Article

Known and putative mechanisms of resistance to EGFR targeted therapies in NSCLC patients with EGFR mutations—a review

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Abstract: Lung cancer is the leading cause of cancer related deaths in Canada with non-small cell lung cancer (NSCLC) being the predominant form of the disease. Tumor characterization can identify cancer-driving mutations as treatment targets. One of the most successful examples of cancer targeted therapy is inhibition of mutated epidermal growth factor receptor (EGFR), which occurs in ~10-30% of NSCLC patients. While this treatment has benefited many patients with activating EGFR mutations, almost all who initially benefited will eventually acquire resistance. Approximately 50% of cases of acquired resistance (AR) are due to a secondary T790M mutation in exon 20 of the EGFR gene; however, many of the remaining mechanisms of resistance are still unknown. Much work has been done to elucidate the remaining mechanisms of resistance. This review aims to highlight both the mechanisms of resistance that have already been identified in patients and potential novel mechanisms identified in preclinical models which have yet to be validated in the patient settings.

Keywords: Epidermal growth factor receptor (EGFR); molecular targeted therapy; drug resistance; antineoplastic

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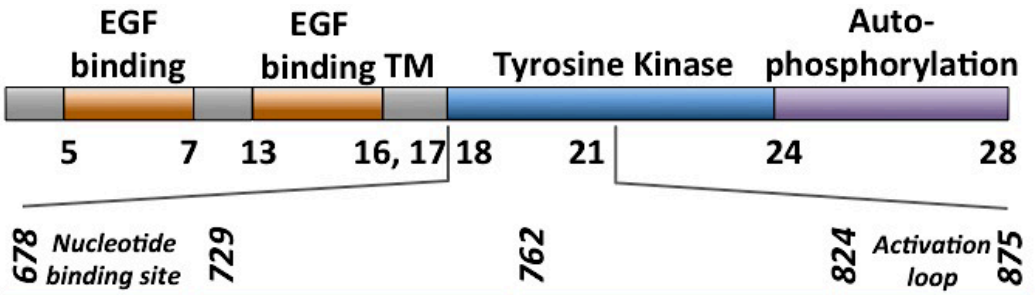
Introduction

Lung cancer is the leading cause of cancer related deaths in Canada (1). In the developed world, non-small cell lung cancer (NSCLC) is the predominant form of the disease, accounting for approximately 85% of cases (2). The advent of molecular profiling has led to the discovery of "driver mutations", targeted therapy, and personalized medicine. Some of the earliest driver mutations discovered and targeted were mutations in the *epidermal growth factor receptor* (*EGFR*) gene (*Figure 1*). EGFR is a receptor tyrosine kinase which, once activated by binding ligand and receptor dimerization, transphosphorylates its cytoplasmic tails, activating cellular signaling pathways such as the phosphoinositide 3-kinase (PI3K)-AKT pathway, the STAT pathway, and the MAPK pathway, ultimately leading to increased cell proliferation, migration, and survival (3-6). Approximately 10-30% of NSCLC patients have activating mutations in *EGFR* (7-9). Targeting EGFR in these patients with activating mutations has shown initial and significant success in the clinic (10,11).

Classical activating mutations, such as the exon 19 deletions and exon 21 L858R substitution, account for approximately 45% and 40% of all *EGFR* mutations, respectively; these two mutations are associated with good responses to EGFR-targeted small molecule inhibitor therapies (11). Initially, these mutations were shown to destabilize the auto-inhibited conformation of the receptor (the normal state of the receptor in the absence of ligand) thus causing constitutive activation of the kinase domain (12-14). More recently, Shan *et al.* (15) reported that the L858R mutation causes a partially disordered state of the EGFR kinase which promotes dimerization and thus aberrant activation. Dixit and Verkhivker (16) recently published the sequence and structure-based computational model which predicted that the L858R mutation synergistically shifts EGFR towards the active state and favours the formation of the asymmetric



EXON 18	EXON 19	EXON 20	EXON 21
G719X (3%)	LREA deletion (45%)	V765A (<1%)	L858R (40%)
	VAIKEL insertion (1%)	T783A (<1%)	L861X (2%)
	L747S (<1%)	V774A (<1%)	T854A (<1%)
	D761Y (<1%)	S784P (<1%)	A871E (<1%)
		T790M *	
		Exon 20 insertion (4%)	
		V769M (<1%)	
		V769M (<1%)	

Figure 1 Missense mutation is represented by the reference amino acid, followed by the residue number, followed by the mutant residue. For summary of somatic mutations found in EGFR. Mutations in green are typically sensitive to EGFR TKIs, those in red are typically resistant. Approximate frequency of occurrence in NSCLC patients of each mutation is shown in parentheses. *T790M is found in ~5% of pre-EGFR TKI treated patient samples and ~60% of post-EGFR TKI treated patient samples. Horizontal numbers represent exons, vertical numbers represent amino acid residues. X indicates when one amino acid has been shown to be replaced by multiple different amino acids, as example, the glycine at position 719 has been shown to be mutated to an alanine, cysteine, or serine. LREA: string of amino-acids leucine, arginine, glutamate, and alanine). VAIKEL: string of amino-acids valine, alanine, isoleucine, lysine, glutamate, and leucine). TM, transmembrane domain; EGFR, epidermal growth factor receptor; TKIs, tyrosine kinase inhibitors [Modified from Sharma *et al.* (3)].

dimer. The L858R activating mutation has also been shown to decrease ATP binding affinity. Yun *et al.* (17) report that this decreased affinity for ATP essentially creates a "therapeutic window", which renders the oncogenic EGFR mutants more easily inhibited by TKIs, as they now have higher binding affinity than, and thus can outcompete ATP.

Over the years, drugs have been developed which specifically target EGFR. One such class is a group of small molecule inhibitors that inhibit the tyrosine kinase domain of EGFR, and are thus referred to as tyrosine kinase inhibitors (TKIs). The first TKIs shown to have clinical benefit were gefitinib and erlotinib (10,11,18). These two TKIs are considered first-generation; they reversibly bind to the tyrosine kinase domain of EGFR (19). First-generation EGFR TKIs have shown significant success clinically in patients with the most common activating *EGFR* mutations. As first-line treatments, EGFR inhibitors have been shown to produce overall response rates (ORRs) of close to 75% in patients who harbor activating mutations in *EGFR* (3,20,21).

Despite this, the vast majority of patients develop resistance to treatment; the median progression free survival (PFS) after treatment with a first generation EGFR TKI in patients with activating mutations is typically less than one year (20-22). Numerous biological mechanisms of acquired resistance (AR) have been elucidated (*Figure 2*), but in up to 30% of patients, the mechanism of resistance remains unknown (23). To date few patients have been cured by an EGFR TKI alone and almost all patients eventually acquire resistance and relapse (21,24). This review aims to give an overview of the most common mechanisms of primary and AR as well as highlight novel, newly emerging theories.

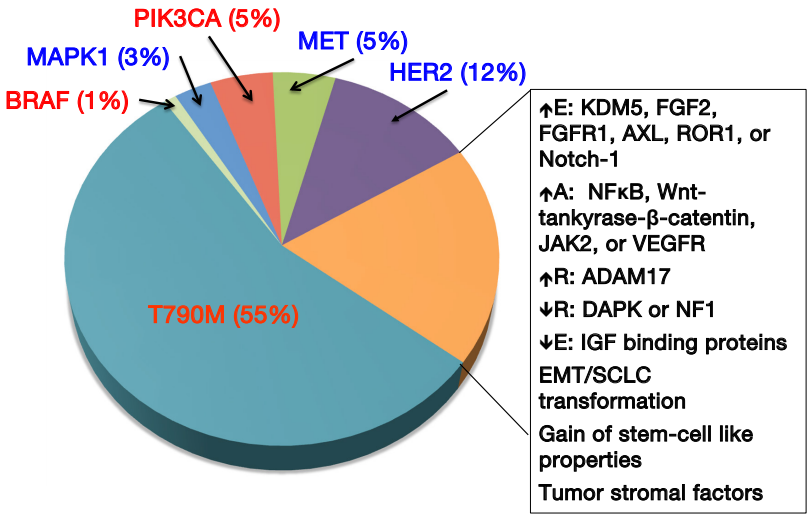


Figure 2 Summary of mechanisms of resistance to first generation EGFR TKIs. Reported occurrence of each mechanism varies somewhat cohort to cohort, thus the shown prevalence rates are approximations. Red text represents mutations, blue text represents amplifications. ↑E, increased expression; ↑A, increased activation; ↑R, up-regulation; ↓R, down-regulation; ↓E, loss of expression.

Primary resistance

EGFR somatic mutations

Depending on the mutation present in *EGFR*, tumors exhibit differential TKI sensitivities. While the most common EGFR-activating mutations, L858R and exon 19 deletion, typically confer sensitivity to EGFR TKIs, other primary EGFR mutations can confer resistance. Exon 20 insertions or duplications, which account for approximately 4-9% of EGFR mutations, appear to be resistant to EGFR inhibitors *in vivo*, despite the fact that these mutations appear to also be activating mutations, at least *in vitro* (25-33). Most of these insertions occur between amino acids 767 to 774 (31). The crystal structure of the exon 20 D770_N771insNPG *EGFR* mutant revealed that the ATP-binding pocket is unaltered, thus EGFR is activated without increasing its affinity for EGFR TKIs (34). Interestingly, loss of these activating *EGFR* mutant genes has been reported *in vitro*, which leads to a decrease in addition to EGFR signaling, gained addiction to both HER2/HER3 and PI3K/AKT signaling, and thus AR to EGFR TKI (35). Other, much less frequent, primary *EGFR* mutations such as G719X and L861X, have been reported (*Figure 1*) (36,37).

Although recognized mainly as a mechanism for AR, another *EGFR* exon 20 mutation, T790M, has also been associated with primary resistance. This mutation is within the gatekeeper residue, and restores the L858R mutant receptors affinity for ATP to wild-type levels, thus decreasing the effect of TKIs (38). Biochemical studies have demonstrated synergistic kinase activity and transformational potential when T790M is concurrently expressed with a TKI-sensitizing, EGFR-activating mutation (39,40).

Minor clones with the T790M mutation have been identified in treatment-naive tumors that contain classic sensitizing mutations. While this mutation has low allelic frequencies in treatment-naive tumors, pressure from TKIs may select for enriched growth of these T790M clones, leading to overall AR. As allelic dilution most likely obscures the detection of *de novo* T790M mutations via conventional Sanger sequencing methods, higher sensitivity assays such as high-performance liquid chromatography, mass spectrometry, locked nucleic acid PCR techniques and next generation sequencing have been suggested as alternate screening methods (41-47). Recent studies using these more sensitive techniques have reported T790M mutations in 35%, 38%, and 79% of *EGFR*-mutant, NSCLC pretreatment specimens (48-50). Interestingly, Rosell *et al.* (48) reported that low levels of BRCA-1 negates the desensitizing effects of the T790M mutations and is associated with longer PFS to erlotinib. Conversely, high levels of BRCA-1 lead to increased DNA damage repair capacity and thus *de novo* resistance.

EGFR germ line polymorphisms associated with primary resistance

T790M

This mutation has also been identified rarely in patients as a germline polymorphism; it has been identified in 0.5% of never smoker-lung cancer patients' blood samples (51). Furthermore, the T790M mutation has also been putatively associated with familial cancer syndromes (52). In short, the proband's mother, maternal grandfather and great uncle all succumbed to bronchioloalveolar carcinoma in their 60's and 70's. Furthermore, three out of the four siblings, including the proband, also developed lung cancer; two of these individuals (including the proband) failed to respond to gefitinib treatment, alone or in combination with chemotherapy. The third sibling was only recently diagnosed at the time of the referenced publication, thus their cancer treatment and subsequent response were not reported. Tumor specimens were available from two of the siblings (five independent primary tumors from the proband and a biopsy from metastatic disease from a brother). *EGFR* sequencing identified the T790M mutation in all tumors in a 1:1 ratio with the wild-type allele. Three of the five tumors from the proband had additional EGFR somatic mutations that typically respond to EGFR TKI therapy (two with L858R, one with delL747_T751); the biopsies from the remaining two primary tumors revealed no additional mutations in *EGFR*. The biopsy from the brother's metastatic lesion also harbored the G719A *EGFR* mutation, which typically confers sensitivity to EGFR TKI therapy. Most intriguingly, the T790M mutation was also present in the germline (measured from peripheral blood mononuclear cells) of both individuals as well as their other two siblings (52). In the report by Girard *et al.* (51), no response information to EGFR TKI was reported.

V843I

In 2008, there was a care report about a woman with a family history of lung cancer (father and a brother) who was diagnosed with multiple adenocarcinomas that exhibited either L858R or L861Q *EGFR* mutations as well as a rare germline *EGFR* mutation, V843I. Three of her four remaining siblings were sequenced, two of whom also harbored the germline mutation, neither of whom had developed lung cancer despite their advanced age (67 and 72 years of age) (53). Another report was published in 2011 on a family with a history of cancer where four of the family members exhibited the germline V843I mutation (54). Three of these family members developed lung cancer, and all of them had the *EGFR* somatic L858R mutation. Only the proband underwent EGFR TKI therapy, however they did not respond to either gefitinib or erlotinib. The most recent report of this germline variation was in 2013, which described the first Caucasian patient with this mutation as well as the first patient without concomitant additional known *EGFR*-activating mutation (55). This patient did not respond to erlotinib and their tumors continued to grow rapidly while on this treatment. Modeling analysis of V843I suggests that ATP and TKI affinities for EGFR are not affected by this mutation; the mechanism of action for a possible germ line predisposition of V843I to develop lung cancer remains unknown. Matsushima *et al.* (56) demonstrated that the V843I mutation increased the phosphorylation of EGFR and downstream signaling proteins compared to wild type EGFR, especially when induced by EGF, suggesting a potentially oncogenic role for this mutation. Furthermore, they demonstrated that the double V843I/L858R mutant did not have increased phosphorylation levels, however the double mutant was resistant to erlotinib, gefitinib, afatinib and dacomitinib. Finally, structural modeling suggests that TKI binding to EGFR would be sterically hindered by Arg841 in the V843I/L858R double mutant (56).

Other genetic polymorphisms

BIM

Despite our furthered understanding of the sensitizing effects that various *EGFR* mutations have to TKIs, patients with identical mutations can demonstrate a spectrum of responses. One explanation for this variability in responses lies within the apoptotic machinery. Recent studies have demonstrated up-regulation of BIM in response to EGFR TKIs in mutant cell lines, which correlated with apoptotic response. *EGFR*-mutant patients with low BIM expression prior to treatment exhibited less tumor shrinkage and shorter PFS after TKI therapy (57-61). Variances in BIM expression levels have been suggested to be due to a genetic polymorphism in BIM, leading to alternative splicing and altered function (58,59,62,63). Clinically, the *BIM* deletion polymorphism has been reported in 12.9% of East Asian individuals. Furthermore, patients with NSCLC who harbor this *BIM* polymorphism exhibit significantly inferior responses to EGFR-TKI treatments compared to wild-type *BIM* counterparts (64). Indeed, Nakagawa *et al.* (64) demonstrated sensitization in EGFR-TKI resistant cell lines that harbor BIM polymorphisms by combination therapy

with HDAC inhibitor vorinostat. Recent results from the randomized phase III EURTAC trial demonstrated that high BIM expression prior to treatment was a marker of longer PFS (HR =0.49; P=0.0122) and overall survival (HR =0.53; P=0.0323) (65). As such, BIM appears to act as both a biomarker and mediator of TKI-induced sensitivities in several oncogene-driven cancers.

Acquired resistance (AR)

Secondary EGFR mutations

The earliest reported mechanism of resistance to TKIs in *EGFR*-mutant NSCLC is the T790M mutation (see previous section on primary resistance), which accounts for approximately 50-60% of cases with AR to EGFR TKI therapy (24,66-69). Despite the multiple avenues of enhanced oncogenicity, tumors harboring T790M mutations often exhibit surprisingly slow growth rates (70). A retrospective study examining T790M status on rebiopsy specimens from 93 patients with *EGFR*-mutant lung cancer and AR to TKIs found that T790M patients had a better prognosis. Furthermore, lack of T790M at time of rebiopsy was associated with a poorer performance status at progression, earlier development of new metastatic disease sites, as well as shorter post-progression survival (24).

Other secondary mutations in *EGFR* linked to AR have also been identified such as D761Y, T854A, and L747S. However, the structural basis for how these mutations confer resistance remains unknown (71-73).

Gene copy alterations of alternative pathways

MET

Amplification of the *MET* gene is considered one of the more common causes of AR in *EGFR*-mutant NSCLC. Heterodimerization of MET and ERBB3 leads to sustained activation of the PI3K/AKT signaling pathway, bypassing the inhibition of EGFR conferred by TKIs (74). Initial reports suggested that *MET* amplification accounted for approximately 22% of AR cases, independent of T790M status. However, two recent studies, each testing 37 patients with AR to EGFR TKIs for MET amplification by FISH, suggest that this prevalence is closer to 5% (44,75). This discrepancy between studies may be in part due to technical difficulties in identifying this genetic alteration in clinical samples. The initial studies with the higher reported percentage of *MET* amplification used several methods of assessment such as array comparative genomic hybridization (aCGH), quantitative real-time PCR, as well as FISH. On its own, FISH is the most widely acceptable technique in clinical laboratories, however technical difficulties arise due to both *MET* and *EGFR* being on chromosome 7. Furthermore, polysomy of chromosome 7 is common in NSCLC, particularly in samples with *EGFR* activating mutations (76). As such, it's been suggested that new clinical protocols to distinguish meaningful *MET* amplification and copy number gain from underlying polysomy in both *EGFR*-mutant and wild-type lung cancers, is required. Aberrant activation of MET and subsequent AR has also been reported via excessive hepatocyte growth factor secretion, the natural ligand for MET (77,78). MET-amplification may not be solely a mechanism of AR but also an inherent event. Low frequencies of MET-amplified subclones have been identified in treatment naive specimens (79). Similar to the development of AR in tumors with low frequencies of T790M, the dominant mechanisms of AR at the time of disease progression in the majority of these cases has been *MET* amplification (80). Recent and on-going attempts to overcome AR due to overriding EGFR inhibition via aberrant MET signaling is to inhibit both receptors simultaneously (80-83). Overall, there is reasonable rationale for clinical trials to evaluate MET inhibitors in patients who developed AR to EGFR TKI therapy via MET amplification mechanism.

HER2 amplification

Recently, amplification of *HER2* has been reported in three of 26 (12%) *EGFR*-mutant NSCLC patients who have AR to TKIs. Similar to *MET*, it is believed that *HER2* is able to signal parallel to inhibited EGFR and thus reactivate common downstream signaling pathways (84).

MAPK amplification

Due to *KRAS* mutations' associations with primary resistance to EGFR inhibitors, recent studies have focused on RAS/MAPK signaling as potential mechanisms of AR (85). *KRAS* mutations themselves are known to be mutually exclusive with *EGFR* mutations in patients. Thus, despite their role in primary resistance, no *KRAS* mutations have been identified in *EGFR* mutant patients with AR (75,85,86). However, Ercan *et al.* (87) identified MAPK1 amplification in an erlotinib-resistant *EGFR*-mutant NSCLC patient. The investigators further demonstrated that a mechanism of resistance to the irreversible EGFR TKI WZ4002 was increased ERK signaling due to amplification of MAPK or down regulation of negative

regulators of ERK signaling. This resistance was overcome by inhibition of MEK or ERK and prevented the development of subsequent resistance.

Mutations in downstream effector molecules of EGFR

PIK3CA mutations

Alternative to parallel pathways being activated, downstream effector molecules of the EGFR signaling pathway have also been reported to be mutated, leading to AR (76). *PIK3CA* mutations have been reported in 5% of *EGFR*-mutant patients who have AR and preclinical studies demonstrate the ability of these mutations to confer resistance via activation of downstream AKT (88). PI3K phosphorylates PIP2 to PIP3; *PTEN (*phosphatase and tensin homolog), reverses this phosphorylation. The loss or decreased expression of PTEN has also been linked to AR (89,90).

BRAF mutations

A recent retrospective study identified point mutations in *BRAF* in two out of 195 (1%) lung cancer patients with AR to EGFR TKIs. The investigators further confirmed *BRAFs* potential role in AR by inducing ectopic expression of mutant BRAF in drug-sensitive *EGFR*-mutant cells, inducing resistance to EGFR TKIs. The addition of a MEK inhibitor was able to overcome induced resistance (86).

Epigenetic and other mechanisms

Epigenetic

Although the genetic basis for acquiring TKI resistance has been well established, a number of recent observations reveal a reversible epigenetic mechanism of drug resistance. Firstly, genetic mechanisms alone cannot account for the high prevalence of TKI-resistant tumors. Secondly, many NSCLC patients who previously developed TKI resistance respond to TKI again after being off the drug for a period of time. Such a phenomenon indicates that acquired TKI resistance might not require a permanent genetic alteration. Thirdly, there is still a significant proportion of TKI resistant tumors that do not harbor any known genetic alterations and activation of alternative signaling pathway. Finally, tumors exhibit not only genetic but also epigenetic heterogeneity within cell populations (91,92).

Epithelial-to-mesenchymal transition (EMT)

EMT, as the name suggests, is a cellular phenotypic change. It can be characterized molecularly by a loss of epithelial markers such as E-cadherin, and a gain of mesenchymal markers, such as vimentin (93). At the cellular level, EMT leads to enhanced motility, invasiveness, and *in vitro* EGFR TKI resistance (94-96). EMT has also been identified in subsets of clinical EGFR TKI-resistant specimens. Despite the growing evidence that EMT may play a role in resistance to treatments, the underlying biology of this change and specific mechanisms of resistance remain unknown (75). Recent work demonstrated the efficacy of blocking ERK1/2 in preventing EMT in lung cancer cells and enhancing their sensitivity to EGFR TKIs. By inhibiting MEK1/2 (MAPKK1/2), an epithelial phenotype was promoted and maintained in NSCLC cells despite exogenous stimulation by TGF-beta. Furthermore, cells that exhibited *de novo* or AR to gefitinib demonstrated decreased cell migration and enhanced sensitivity to the EGFR TKI when MEK was inhibited long enough to trigger changes in EMT marker expression (97).

Histological transformation

Several studies have reported the histological transformation to small cell lung cancer in *EGFR* mutant NSCLC patients with acquired EGFR TKI resistance, accounting for resistance in possibly up to 3% of the patients. Interestingly, the conversion to SCLC was associated with sensitivity to standard SCLC treatment while the original *EGFR* mutation was still maintained in the tumor (75,98). The mechanism underlying this histological transformation still remains unknown.

AXL activation

AXL is a tyrosine kinase receptor which induces cell proliferation, migration and invasion in cancer. Recently, several groups reported that activation of AXL signaling pathway may confer TKI resistance in *EGFR* mutant NSCLC (99,100). Activation of AXL signaling pathway can occur through overexpression of AXL or its ligand GAS6. Small-molecule AXL inhibitors, MP-470 and XL-880 were able to restore the TKI sensitivity in TKI resistant NSCLC cells. Forced overexpression of AXL in TKI sensitive NSCLC cells can confer TKI resistance. These investigators also found an association between the overexpression of AXL and vimentin, a marker of EMT in the TKI resistant NSCLC cells. In their exploratory analysis of patient samples, approximately 20% of EGFR TKI resistant NSCLC patients were found to have tumors with upregulated AXL, GAS6 and vimentin.

NF-κB activation

NF-κB is an important transcription regulator of the genes that controls cell proliferation and cell growth, including tumor growth. Bivona *et al.* (101) reported previously that activation of NF-κB signaling pathway can confer TKI resistance in *EGFR* mutant NSCLC cells. The investigators introduced a shRNA library to target >2,000 cancer relevant genes in the TKI insensitive H1635 NSCLC cell line. This line had an *EGFR* mutation, but no other identifiable mutations or activation of alternative signaling pathways that could confer insensitivity to EGFR TKI. Among the screen hits conferring TKI sensitivity in H1635, 18 target genes were linked to the NF-κB signaling. Inhibition of NF-κB signaling could enhance TKI sensitivity in H1635 and other *EGFR*-mutant NSCLC cells, and they reported that higher NF-κB activation state was correlated with worse PFS and decreased overall survival in *EGFR*-mutant NSCLC patients treated with TKI. However, a recent clinical study of the combination of PF-3512676, an inhibitor for toll-like receptor 9 which activates NF-κB, and erlotinib did not increase PFS as compared to erlotinib alone in patients with advanced recurrent *EGFR*-mutant NSCLC patients (102).

IGF1-R and KDM5A activation

Sharma *et al.* (103) reported that a subpopulation of NSCLC tumors developed reversible TKI resistance by engaging the IGF1-R signaling pathway and an altered chromatin state due to a histone demethylase, KDM5A. These TKI resistant cells had upregulated IGFBP-3, KDM5A and increased phosphorylation of IGF-1R. In this subpopulation, IGF1-R inhibitor, depletion of KDM5A or histone deacetylases (HDACs) could markedly suppress the TKI-resistant outgrowth of NSCLC cells in combination with TKI by restoring the TKI sensitivity of TKI resistant cells. Furthermore, inhibition of IGF1-R could lead to decreased KDM5A expression and restoration of H3K4 methylation, suggesting a direct link between IGF-1R signaling pathway and KDM5A function. Altogether, the authors demonstrated that a transient altered chromatin state could potentially mediate TKI resistance in NSCLC. Unfortunately, a recent randomized Phase II study concluded that the combination of IGF1-R inhibitor (R1507) with erlotinib did not provide any PFS or survival advantage over erlotinib alone in unselected NSCLC patients (104). A clinical study to evaluate the combination of erlotinib and HDAC inhibitor, SNDX-275 *vs.* erlotinib alone in treatment of NSCLC patients has just been completed (NCT000602030), but the results have not been reported.

Other alternative signaling pathway activation

Recently many more signaling pathways have been reported to mediate resistance to EGFR TKI in NSCLC models, but as yet lack evidence for efficacy in patients. These pathways include: activation of Wnt-tankyrase-β-catenin pathway; reduced expression of NF1; downregulation of DAPK through DNA methylation of its CpG island; overexpression of FGF2 and FGFR1 in FGF2-FGFR1 autocrine pathway; upregulation of ADAM17 in heregulin-HER3 autocrine loop; activation of JAK2-related signaling pathway; overexpression of ROR1 caused by NKX2-1; activation of VEGF signaling pathway in stromal cells; overexpression of Notch-1 and its enhancement of EMT; loss of IGF binding proteins; acquisition of stem-cell like properties; and involvement of tumor stroma and cancer-associated fibroblasts derived from EGFR-TKI-resistant tumors (105-118). Many of these pathways have been known to be relevant in cancer development and progression.

Current clinical strategies to overcome AR

When patients relapse secondary to AR, alternative treatment strategies are desired. There is increasing evidence to support patient tumor rebiopsy upon development of resistance to determine the optimal second-line treatments; some cancer centers and clinical trials are already implementing this strategy (119,120). For various cancer sites, rebiopsy is a fairly simple procedure. For lung cancer patients, however, rebiopsy is often a highly invasive procedure, and in many cases, there is a difficult choice of which of multiple metastatic sites should be considered for biopsy. Some patients who develop initial resistance to an EGFR TKI respond again upon a second challenge, after a defined period of a TKI drug holiday (121-124). Song *et al.* reported that, based on multiple studies, over 50% of patients who progressed on a first line EGFR TKI and then stopped the TKI treatment, benefited from a subsequent second course of the same EGFR TKI (125). There is currently a poor understanding of the mechanisms of reversal of resistance conferred by such a drug holiday.

Optimal therapies have not been established for the majority of *EGFR*-mutant lung cancer patients who develop disease progression after merely 10 to 14 months on TKIs (20,24,126). *Table 1* summarizes the results of clinical trials to date using second and third generations TKIs that were

Table 1 Response rates to second and third generation EGFR TKIs in clinical trials

Agent	Study	Prior chemo-therapy	Prior EGFR TKI therapy	EGFR mutation required	No. of pts with EGFR mutation	ORR (in all pts, %)	No. pts with T790M	ORR (in T790M+ pts, %)
Second generation TKI								
Neratinib	NCT00266877 (127)	Yes & no	Yes & no	No	91	3	12	0
Afatinib	LUX-Lung 3 (128)	No	No	Yes	345	56	NR	NR
	LUX-Lung 6 (129,130)	No	No	Yes	242	67	NR	NR
	LUX-Lung 2 (131)	Yes & no	No	Yes	129	61	1	NR
	LUX-Lung 1 (132)	Yes	≥12 weeks E/G	No	62	7	4	NR
	LUX-Lung 4 (133)	Yes	≥12 weeks E/G	No	56	8	2	NR
	LUX-Lung 5 (134)	Yes	≥12 weeks A	No	NR	32	NR	NR
Afatinib + cetuximab	NCT01090011 (135)	Yes	Yes	Yes	126	29	71	32
Dacomitinib	ARCHER 1009 (136)	Yes	Yes	No	47	11	NR	NR
	BR26 (137)	Yes	Yes	No	157	7	NR	NR
Third generation TKI								
AZD9291	NCT01802632 (138)	Yes	Yes	Yes	199	55	132	64
HM61713	NCT01588145 (139)	Yes	Yes	Yes	93	17	27	66
CO-1686	NCT01526928 (140)	Yes	Yes	Yes	88	58*	55	58*

pts, patients; EGFR, epidermal growth factor receptor; NR, not reported; ORR, objective response rate; E/G, erlotinib/gefitinib; A, afatinib; TKI, tyrosine kinase inhibitor; *, ORR was calculated from phase 2 which included only T790M+ pts.

supposed to overcome AR. Second-generation EGFR TKIs have been developed to overcome resistance, however, results from clinical trials have not been as promising as was anticipated.

Second-generation EGFR TKIs form irreversible covalent bonds with the ATP-binding site of EGFR as well as other members of the HER family of receptors (excluding Her3). Neratinib (HK1-272) did not show good response rates (RR) in patients with T790M mutations thus further development was halted (127). Afatinib (BIBW2992) has been investigated as a second- and third-line treatment in patients who have AR to first-generation EGFR TKIs (LUX-Lung 1, 4, and 5 program) and as a first-line treatment in *EGFR*-mutant patients (LUX-Lung 2, 3, 6 and 7). Thus far, afatinib has been shown to improve the disease control rate and prolong PFS in both LUX-Lung 1 and 2 (131,132). The LUX-Lung 4 trial demonstrated a modest benefit of afatinib as a third- or fourth-line treatment for patients who had previously progressed while receiving erlotinib and/or gefitinib (133). The LUX-Lung 5 trial demonstrated the benefit of combining paclitaxel with afatinib after patients with AR to gefitinib and/or erlotinib progress on afatinib monotherpy (134). Dacomitinib (PF-00299804), another second-generation, irreversible pan-HER TKI, has shown activity against NSCLC cell lines that harbor the T790M mutation. Dacomitinib efficacy was studied in two phase II trials. The first was to evaluate benefit (compared to erlotinib) after failure of one or two chemotherapy regimens, the second compared its benefit as a second- or third-line treatment in patients with advanced NSCLC after failure of at least one prior chemotherapy regimen and prior treatment with erlotinib (141,142). While the results of these two studies seemed initially promising, two randomized phase 3 studies, the ARCHER 1009 trial and the NCIC CTG BR.26 trial, failed to meet their objectives (136,137). The ARCHER 1009 trial did not demonstrate any statistically significant PFS in advanced NSCLC patients treated with dacomitinib compared to erlotinib in the second- and third-line therapy of advanced NSCLC (136). The NCIC CTG BR.26 trial, which included patients with advanced NSCLC who failed previous standard therapy with both chemotherapy and an EGFR TKI, failed to demonstrate significant prolongation of overall survival in those treated with dacomitinib versus placebo, though there was significant improvement in response rate, PFS and time to symptom deterioration in

patients with *KRAS* WT NSCLC (137). In neither of these trials were patients selected specifically for the presence of the T790M mutation.

Recent studies have demonstrated the benefit of combining therapies in overcoming resistance that arises through secondary mutations in the driver oncogene. In both cell line-derived and transgenic mouse models harboring T790M mutations, concurrent administration of the irreversible EGFR TKI, afatinib, and EGFR monoclonal antibody, cetuximab, resulted in dramatic tumor shrinkage (143). A phase I/II trial investigating the same drug combination in NSCLC patients with *EGFR* mutations and AR to EGFR TKIs demonstrated responses in 40% of patients (135,143). The mechanisms underlying the synergistic effect of this combination appear to be a dramatic inhibition of both phosphorylated EGFR and total EGFR. In contrast, afatinib appears to affect only phosphorylated EGFR and cetuximab appears to only affect the total EGFR protein expression (143). Meador *et al.* (144) developed resistance to the afatinib/cetuximab combination in PC-9/BRc1- (exon19 deletion/T790M mutant *EGFR* NSCLC cell line) derived xenografts and found that this occurred via the additional amplification of the *EGFR* gene. They further demonstrated sensitivity in this resistant model to the third-generation EGFR TKI AZD9291.

Third-generation EGFR TKIs specifically target both activating mutations and T790M mutations in *EGFR*. These agents seem promising; early results from phase I trials on three 3rd generation EGFR TKIs were presented at the 2014 ASCO Annual Meeting. The first study of HM61713 in advanced NSCLC patients with EGFR mutations who had failed previous EGFR TKIs (NCT01588145) demonstrated disease control rates of 76.5% when treated <4 weeks, and 73.1% when treated ≥4 weeks; 18 of 27 patients carrying T790M mutations showed a decrease in the target lesion sizes (139). The use of AZD9291 in EGFR mutant NSCLC patients (NCT01802632) resulted in (unconfirmed) response rates of 64% in 89 patients with T790M (with disease control in 96%) and only 23% in 43 patients without T790M mutations documented. Importantly, RECIST responses were observed at all dose levels and in brain metastases (138). For the 3rd generation EGFR TKI, CO-1686 (NCT01526928), preliminary results found that, of nine patients carrying T790M mutations, six demonstrated partial responses (PRs), two achieved stable disease, and the final patient achieved PR after transitioning to the HBr form of CO-1686 (140). Despite these promising, early clinical results, resistance to at least one of these third-generation TKIs, CO-1686, has already been demonstrated by an EMT mechanism (145).

Summary

Targeting EGFR in NSCLC patients with activating mutations holds great promise, however AR remains a currently insurmountable hurtle. Mechanisms behind AR have been identified in patients, such as secondary mutations within *EGFR*, activation of alternate proteins that are downstream of EGFR signaling or activation of proteins that feed into the EGFR signaling cascade. Further mechanisms of AR have been identified in cell lines and remain to be observed in patients. Novel treatment regimens of EGFR TKIs in combination with therapies that target EGFR in different ways or that target alternate proteins are being attempted to overcome known mechanisms of resistance. Third generation EGFR TKIs are being developed in the hopes of overcoming the most common mechanisms of resistance, T790M; to date, the results are preliminary but excitingly optimistic.

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