Review Article on Mechanisms of Resistance to EGFR-targeted Therapy

Impact of concurrent genomic alterations in epidermal growth factor receptor (*EGFR*)-mutated lung cancer

Beatrice Gini[1,2], Nicholas Thomas[1,2], Collin M. Blakely[1,2]

[1]Department of Medicine, [2]Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, California, USA

Contributions: (I) Conception and design: All authors; (II) Administrative support: B Gini; (III) Provision of study materials or patients: None; (IV) Collection and assembly of data: N Thomas, B Gini; (V) Data analysis and interpretation: CM Blakely, B Gini; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Collin M. Blakely. Department of Medicine, University of California, San Francisco, California, USA; Helen Diller Family Comprehensive Cancer Center, University of California, 550 16th Street, San Francisco, CA 94158, USA. Email: collin.blakely@ucsf.edu.

Abstract: Comprehensive characterization of the genomic landscape of epidermal growth factor receptor (*EGFR*)-mutated lung cancers have identified patterns of secondary mutations beyond the primary oncogenic *EGFR* mutation. These include concurrent pathogenic alterations affecting p53 (60–65%), RTKs (5–10%), PIK3CA/KRAS (3–23%), Wnt (5–10%), and cell cycle (7–25%) pathways as well as transcription factors such as MYC and NKX2-1 (10–15%). The majority of these co-occurring alterations were detected or enriched in samples collected from patients at resistance to tyrosine kinase inhibitor (TKI) treatment, indicating a potential functional role in driving resistance to therapy. Of note, these co-occurring tumor genomic alterations are not necessarily mutually exclusive, and evidence suggests that multiple clonal and sub-clonal cancer cell populations can co-exist and contribute to EGFR TKI resistance. Computational tools aimed to classify, track and predict the evolution of cancer clonal populations during therapy are being investigated in pre-clinical models to guide the selection of combination therapy switching strategies that may delay the development of treatment resistance. Here we review the most frequently identified tumor genomic alterations that co-occur with mutated *EGFR* and the evidence that these alterations effect responsiveness to EGFR TKI treatment.

Keywords: Lung cancer; oncogenes; mutations; therapy

Submitted Dec 21, 2019. Accepted for publication Mar 12, 2020.
doi: 10.21037/jtd.2020.03.78
View this article at: http://dx.doi.org/10.21037/jtd.2020.03.78

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide (1). Research into the complex genetic basis of lung cancer initiation and progression has yielded novel treatments that have revolutionized lung cancer management (2). The expansion of clinical sequencing efforts has led to the identification of several novel oncogenic driver alterations (3,4), and has spurred the development of small molecule tyrosine kinase inhibitors (TKIs) aimed at suppressing signaling pathways downstream (2). Activating mutations in the epidermal growth factor receptor (*EGFR*) gene have been identified as oncogenic driver mutations in approximately 15% of lung adenocarcinoma (LUAD) patient tumors in Western populations and in over 50% of patients of East Asian descent and represent the most frequent targetable genetic alterations identified in lung cancer (4-7). The two most common EGFR-activating alterations are in-frame deletions in exon 19 (E746-A750del) and amino acid substitution in exon 21 (L858R), which together account for >90% of known EGFR driver mutations (6,8).

With the identification of these driver alterations, a new paradigm for lung cancer treatment emerged. Tumor biopsy and sequencing to identify a driver mutations followed by TKI treatment (e.g., erlotinib, gefitinib,

afatinib, osimertinib in the case of EGFR) replaced first line platinum-based chemotherapy, which had limited clinical efficacy with a median overall survival (OS) <12 months and a 5-year survival rate <1% (9). Unfortunately, these practice changes resulted in only modest improvements in progression-free survival (PFS) over platinum therapy for patients with EGFR mutations, ultimately limiting long-term OS (10). Even with a targeted therapy approach, the clinical success of treatment with TKIs is almost uniformly limited by the development of drug resistance and progression emerges after a median of 8–18 months (11,12).

The *EGFR* T790M on-target resistance mutation was first identified as a common mechanism of resistance to first- and second-generation EGFR TKIs (13,14). Similarly, although less frequent, the *EGFR* C797S mutation has emerged as an on-target resistance mutation to the 3rd generation EGFR TKI osimertinib (15). Additional studies profiled and identified tumor genetic alterations that are pre-existing or acquired after TKI treatment and can bypass EGFR signaling pathway inhibition, promoting resistance to EGFR-directed therapy (5,16-20). Beyond direct tumor sequencing, liquid biopsies can capture the heterogenous mutational landscape of metastatic tumors at different sites. Analysis of circulating tumor DNA (ctDNA) from patients with advanced *EGFR*-mutated non-small cell lung cancer (NSCLC) point to co-occurring tumor genomic alterations and tumor mutational heterogeneity as a common feature of EGFR-mutant lung cancers (5,21). This may ultimately contribute to the limited depth and duration of response to EGFR inhibitors. Herein, we review our current understanding of how concurrent tumor genetic alterations limit response and lead to resistance to EGFR-targeted therapy.

Types of concurrent resistance mutations by pathway in lung cancer

EGFR-dependent co-alterations

Acquired resistance to EGFR TKI treatment can occur through the acquisition or selection of pre-existing TKI resistance mutations (*Table 1*). In 60% of EGFR-mutant NSCLC patients treated with 1st (erlotinib, gefitinib) and 2nd (afatinib) generation EGFR TKIs, acquisition of resistance is triggered by the substitution of threonine to methionine at position 790 in exon 20 (T790M) which impedes drug binding and increases ATP affinity in the EGFR ATP-binding pocket (11,22). Third generation EGFR TKIs have been developed to overcome T790M-mediated resistance. Osimertinib (AZD9291) (68) selectively targets both canonical EGFR activating mutations as well as the T790M resistance mutations by covalently binding the C797 residue in the ATP pocket of mutant EGFR (69). Patients whose tumors harbored the EGFR T790M mutation and were treated with osimertinib in the second line setting had a median PFS of 9.6 months (68). Furthermore, osimertinib resulted in significantly improved PFS compared to platinum-based chemotherapy (23). In the FLAURA study, osimertinib was tested as first-line therapy in comparison to 1st generation EGFR TKIs (gefitinib or erlotinib), and demonstrated significantly improved PFS (18.9 *vs.* 10.2 months) and OS (38.6 *vs.* 31.8 months), as well as a better safety profile (10,23). Although these results supported the use of osimertinib as first-line therapy in advanced-stage *EGFR*-mutated NSCLC patients, intrinsic and acquired osimertinib resistance can occur through tertiary EGFR mutations including C797 (7% of patients treated with front-line osimertinib), or the more rare G796, L792, L718, G719, G724 residue substitutions and additional exon 20 mutations (23-34,70). These mutations can co-exist at low frequency with sensitizing EGFR mutations and sterically interfere with the binding of the drug to the active site (23).

EGFR-independent co-alterations

A. P53 pathway

TP53 is the most frequently altered gene in human cancers (71). *TP53* is a tumor suppressor whose gene product is responsible for the induction of cell-cycle-arrest or apoptosis programs in response to cellular stress, including stress induced by oncogene activation (72). When *TP53* is mutated and this crucial cell programming is lost, unchecked cell proliferation can occur leading to carcinogenesis (73). *TP53* codes for a 393-aa protein composed of three domains: one trans-activation domain, which is a target of post-translational regulation, one DNA-binding domain (DBD) (spanning exons 5–8, aa 102–292) accounting for most of its tumor suppressor activity, and a C-terminal domain responsible for negative protein regulation through oligomerization (74). p53-DNA interactions are mediated by loops L2 (aa 163–195) and L3 (aa 236–251) in the DBD (75). Many *TP53* mutations affect the DBD and generate a mutated protein with a dominant negative effect (76). In addition, *TP53* mutations with gain of function activities have been

Table 1 Concurrent functional gene alterations in EGFR-mutant NSCLC

Co-alteration	Gene	Type		Frequency	Clinical context	Stage	refs
		Alteration	Result				
EGFR dependent	*EGFR*	SNVs	T790M	50%	1st, 2nd gen. TKIs, > post-TKIs	Met.	(11,22,23)
			C797X	10–26%	3rd gen. TKI, 1st/2nd line	Met.	(23-25)
			G796X	Rare	3rd gen. TKI, 2nd line	Met.	(23,25-27)
			L792X	Rare	3rd gen. TKI, 2nd line	Met.	(23,25)
			L718X	Rare	3rd gen. TKI, 1st/2nd line	Met.	(23,25,27,28)
			G719A	Rare	3rd gen. TKI, 2nd line	Met.	(23,25,27)
			G724S	Rare	3rd gen. TKI, 1st line	Met.	(23,26,29-32)
		SNVs ins	Exon 20	Rare	3rd gen. TKI, 1st/2nd line	Met.	(23,28,32,33)
		CNV	Amp	Under study	3rd gen. TKI, 1st line	Met.	(23,34)
EGFR independent							
P53 pathway	*TP53*	SNVs	Mts	60–65%	> post-TKI, 1st, 2nd gen. TKIs	Early/met.	(5,16,35-39)
	MDM2	CNV	Amp	12%	> pre-TKI	Met.	(16,40)
RTKs	*MET*	CNV	Amp	9.9%	> post-TKI, 1st, 2nd, 3rd (1st/2nd line) gen. TKIs	Met.	(5,16,17,23,28,41-46)
	ERBB2	CNV	Amp	8.6–12%	Pre/post-TKI, 1st, 2nd, 3rd (1st/2nd line) gen.TKIs	Met.	(5,16,19,23,28,34)
	PDGFRA	SNVs, CNV	Mts, amp	4.7%	post-TKI?	Met.	(5)
PIK3CA/KRAS pathways	*PIK3CA*	SNVs, CNV	Mts, amp	13%	> post-TKI, 1st, 3rd (1st/2nd line) gen. TKIs	>met.	(5,16,17,23,28,34,47-49)
	KRAS	SNVs, CNV	Mts, amp	4.7%	> post-TKI? 1st, 2nd, 3rd (1st/2nd line) gen. TKIs	Met.	(5,16,17,23,46,47,50-54)
	BRAF	SNV	V600E	3%	> post-TKI, 1st, 3rd (2nd line) gen. TKIs	Met.	(5,16,23,28,34,55)
	NF1	SNVs	Mts	16–23%	Pre/post-TKI	Met.	(5,56)
	mTOR	SNV	E2419K	1 pt	Post-TKI, 1st gen. TKI	Met.	(16)
Wnt pathway	*CTNNB1*	SNVs	Mts	5.3–9.6%	Pre/post-TKI, 1st, 2nd gen. TKIs	>met.	(5,16,39,57-60)
TF	*MYC*	CNV	Amp	10.6%	Pre/post-TKI, 1st gen TKI	Early/met.	(5,61)
	NKX2-1	CNV	Amp	15%–11%	> pre-TKI	Early/met.	(16,39,62,63)
Gene fusions	*FGFR3-TACC3*	Fusion		3–10%	Post-TKI, 1st, 3rd (2nd line) gen. TKIs	Met.	(16,23,34,47)
	RET-ERC1	Fusion		3–10%	Post-TKI, 3rd (2nd line) gen. TKI	Met.	(17,23,34)
	CCDC6-RET	Fusion		3–10%	Post-TKI, 3rd (2nd line) gen. TKI	Met.	(17,23,47)
	NTRK1-TPM3	Fusion		3–10%	Post-TKI, 3rd (2nd line) gen. TKI	Met.	(17,23)

Table 1 (*continued*)

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Co-alteration	Gene	Type		Frequency	Clinical context	Stage	refs
		Alteration	Result				
	NCOA4-RET	Fusion		3–10%	Post-TKI, 3rd (2nd line) gen. TKI	Met.	(17,23)
	GOPC-ROS1	Fusion		3–10%	Post-TKI, 3rd (2nd line) gen. TKI	Met.	(23,64)
	AGK-BRAF	Fusion		3–10%	Post-TKI, 1st, 3rd (2nd line) gen. TKI	Met.	(16,23,65)
	ESYT2-BRAF	Fusion		3–10%	Post-TKI, 3rd (2nd line) gen. TKI	Met.	(23,47)
	SPTBN1-ALK	Fusion		1 pt	Post-TKI, 3rd (2nd line) gen. TKI	Met.	(23)
	PLEKHA7-ALK	Fusion		Rare	Post-TKI, 3rd (2nd line) gen. TKI	Met.	(23,66)
	EML4-ALK	Fusion		Rare	Post-TKI, 3rd (2nd line) gen. TKI	Met.	(23,67)
Cell cycle	*Rb1*	SNVs CNV	Mts, del	9.5–10.3%	> post-TKI, 1st gen. TKI	Early/met.	(16,39)
	CDK4	CNV	Amp	7–10%	Pre/post-TKI, 3rd (1st line) gen. TKI	Met.	(5,23,39)
	CDK6	CNV	Amp	7%	Pre/post-TKI, 3rd (1st/2nd line) gen. TKI	Met.	(5,23,39)
	CCNE1	CNV	Amp	6.9%	Pre/post-TKI, 3rd (1st/2nd line) gen. TKI	Early/met.	(5,23,39)
	CDKN2A	CNV	Del	24.6%	> post-TKI, 1st, 3rd (2nd line) gen. TKIs	Early/met.	(16,39)
	CDKN2B	CNV	Del	20.2%	> post-TKI, 1st gen. TKI	Early/met.	(16,39)

TKI, tyrosine kinase inhibitor; NSCLC, non-small cell lung cancer; TF, transcription factors; SNVs, single nucleotide variants; CNV, copy number variation; mts, mutations; gen., generation; early, early stage; met., metastatic.

described and may contribute to cancer progression and drug resistance (77-79).

TP53 mutations are classified as "disruptive" or "nondisruptive". Disruptive mutations lead to complete or near complete loss of p53 activity and include nonsense and missense mutations in L2 or L3 loops affecting residue polarity and in-frame deletions within L2 or L3 loops (74). All other types of *TP53* mutations are classified as nondisruptive mutations, which cause partial loss of p53 protein function and often associate with gain of function activity (74,80,81).

TP53 mutations frequently occur in NSCLC (ranging from 40–70%) and are commonly found co-occurring with *EGFR* mutations (35-38). A pooled analysis from 4 randomized trials by Ma *et al.*, found *TP53* mutations status to be of no prognostic value in *EGFR*-mutated NSCLC (82). However, when *TP53* mutations were categorized by subtype, nondisruptive *TP53* mutations were identified as an independent prognostic factor of reduced OS in *EGFR*-mutated NSCLC patients (17.8 *vs.* 28.4 months) (74). In another study, *TP53* mutations were classified into "poor" mutations (affecting exon 4, exon 6, mutations of unknown site and multiple mutations) and "good" mutations (exon 5, exon 7, exon 8 and exon 9 mutations), with patients whose tumors harbored *TP53* poor mutations having the worst prognosis in presence of concurrent exon 19/21 mutated *EGFR*. In contrast, patients whose tumors harbored non-exon 19/21 mutated *EGFR* were associated with worse prognosis when "good" *TP53* mutations were identified as co-occurring with the *EGFR* mutation (36).

In a study of cell-free DNA from 1,122 advanced-stage *EGFR*-mutated lung cancer patients, 55% had a co-occurring *TP53* mutation (5) (*Table 1*). In another study, patients with EGFR-mutant lung cancer with any co-occurring *TP53* mutations had a 3-fold risk of disease progression on TKI treatment than *TP53wt* patients (83). Furthermore, *in vitro* studies demonstrated that *TP53* mutant NSCLC cell lines underwent less apoptosis in response to gefitinib when compared to *TP53wt* controls (84).

The clinical significance of co-occurring *TP53* mutations in *EGFR*-mutated NSCLC remains unsettled. Tsui *et al.* found that *EGFR*-mutated patients with *TP53* co-mutations identified prior to TKI treatment exhibited worse OS compared to those without *TP53* mutations (85). Labbé *et al.* found that only *TP53* missense co-mutations were predictive of shorter disease-free survival after surgical resection (86). Other studies found only trends towards poorer PFS and OS when *TP53* co-mutations were identified (83,87). *TP53* co-mutations are frequently clonal in tumors, suggesting early occurrence during tumorigenesis, with a strong selective pressure for *TP53* locus loss of heterozygosity (88). One hypothesis to explain the apparent decrease in OS associated with *TP53* co-mutations is that these mutations may confer higher tolerability to genomic instability, as exemplified by high rates of tumor aneuploidy and somatic mutation burden, potentially promoting a more aggressive tumor phenotype (88).

In addition to *TP53* mutations, amplification of the gene encoding the Mouse Double Minute 2 (MDM2) protein have been identified in 12% of pre-treatment metastatic EGFR-mutant lung cancers. MDM2 mediates proteasome-dependent p53 degradation, and its hyperactivation may lead to loss of p53-tumor suppressive function, potentially contributing to p53-dependent mechanisms of TKI resistance (16,40).

B. RTKs

MET

MET alterations have been found to co-occur with mutant *EGFR* with a frequency of ~10% (5) (*Table 1*). *MET* amplification is a classic mechanism of EGFR TKI resistance presenting in 5% to 22% of cases of acquired resistance (16,20,89). This is thought to be due to its gene location at a fragile site in chromosome 7, which facilitates its amplification by recurrent breaks within chromosomal common fragile sites (90). Subsequently, selection for clones harboring *MET* amplification can occur under drug pressure where MET amplification leads to resistance by maintenance of MAPK/PI3K/AKT signaling (41,91). Recent studies show that concurrent *MET* copy number gains (CNG) in EGFR-mutant NSCLC patients did not affect response to first and second generation of EGFR TKI in the first line setting, except when patients were classified as *MET* amplified (42). This highlights the importance of defining CNG thresholds in the diagnostic practice, which could guide more successful treatment combinations of MET and EGFR inhibitors (41-43). In addition, *MET* amplification has been identified as a common mechanism that drives resistance to first and second line osimertinib treatment in NSCLC patients. Preclinical studies and case reports suggest that this mechanism of resistance can be overcome by combination therapy with crizotinib and osimertinib (17,28,44-46).

ERBB2

ERBB2 belongs to the HER family of receptor tyrosine kinases, which activates the PI3K-AKT and MAPK downstream pathways (23). The most commonly encountered *ERBB2* mutations in lung cancer are in-frame insertions in exon 20, but point mutations along the tyrosine kinase domain have also been identified (92,93). These exon 20 mutations have been demonstrated to be oncogenic drivers and tend to be mutually exclusive with the more common activating mutations in *EGFR* and *KRAS* (94). *ERBB2* amplification, however, have been observed in 12% of *EGFR*-mutated lung cancers at acquired resistance to 1st generation EGFR TKIs gefitinib or erlotinib (19) (*Table 1*). Notably, all of the *ERBB2* amplification positive samples tested were T790M negative, suggesting a distinct mechanism of TKI resistance attributable to ERBB2 (19). This finding was further studied in cellular models of lung cancer where *ERBB2* overexpression (>50-fold above baseline, as per densitometry assessment) in EGFR-mutant NSCLCs conferred resistance to erlotinib (19). *ERBB2* alterations in patients with resistance to first and second line third generation EGFR TKIs have also been described in 2% and 5% of cases respectively, and can co-exist with additional oncogenic *EGFR*, *PIK3CA* and *MET* alterations (28,34). The role of ERBB2 over-expression in reducing third generation EGFR TKIs sensitivity has also been confirmed in preclinical models (46).

C. PIK3CA/KRAS pathways

PIK3CA

Mutations in *PIK3CA* are found in approximately 7% of LUADs (4). These alterations are primarily localized to

the catalytic subunit of the PI3K enzyme, in exons 9 and 20 of the helical and kinase domains of p110 alpha (4). As a result of these mutations, PI3K constitutively activates the AKT-mTOR pathway, triggering tumor cell survival and proliferation (95,96). Concurrent *EGFR* and *PIK3CA* alterations were detected in 13% of metastatic LUAD and were enriched in post-first and second EGFR TKI samples (16) (*Table 1*). In previous clinical investigations, the presence of these co-alterations correlated with poor prognosis but did not impact the efficacy of first-generation EGFR TKI monotherapy (97). In a recent study, longitudinal sampling of seven tumor samples from an individual patient prior to treatment and at disease progression to 1st and 3rd generation EGFR inhibitors highlighted the pre-treatment existence (PIK3CA G106V) and post-therapy enrichment (PIK3CA H1047R) of *PIK3CA* mutations (5). Functional characterization of PIK3CA G106V confirmed its role in promoting tumor cell invasion without affecting sensitivity to EGFR TKI treatment (5). *PIK3CA* mutations identified in NSCLC patients resistant to second line osimertinib treatment occur at a frequency of 4–11% and include: E545K, E542K, R88Q, N345K and E418K with the E545K functionally validated as mediator of resistance (17,34,47-49). Resistance to frontline osimertinib has also been correlated with E453K, E545K (predominant, 4% of cases) and H1047R *PIK3CA* mutations (28).

KRAS/BRAF/NF1

Aberrations in the RAS-MAPK pathway lead to first and second line osimertinib resistance (23,50). In patients with acquired resistance to osimertinib, concurrent KRAS G12S, G13D, Q61R, Q61K and G12D mutations have been reported (17,46,47,51,52). In preclinical models, concurrent *KRAS* mutations resulted in EGFR TKI resistance, which could be overcome using a combination of osimertinib and a MEK inhibitor (50,53,54), suggesting a potentially effective clinical strategy.

Oncogenic *BRAF* mutations are found in 2–3% of LUAD, among which V600E represents 50% of mutations (98-101). Concurrent *BRAF* alterations are present in ~11% of *EGFR*-mutant NSCLC patients (*Table 1*), with the BRAF V600E mutation identified as a mechanism of resistance to osimertinib in ~3% of cases (5,28,34). Tumor cells from patients carrying BRAF V600E resistance co-mutation showed sensitivity to combination of BRAF inhibitor and osimertinib (5,55), suggesting that this may be a viable clinical strategy to overcome resistance.

The *NF1* gene encodes a GTPase-activating protein that down-regulates Ras-signaling through guanosine triphosphate (GTP) hydrolysis, thus acting as a tumor suppressor (102). Primary and acquired resistance of LUAD patients to EGFR TKI has been associated with *NF1* deletion (56). Sensitivity to EGFR TKI was rescued in cells with low NF1 expression by adding a MAP-ERK kinase (MEK) inhibitor (56). In a small EGFR-mutant LUAD liquid biopsy cohort, for which treatment outcome data were available, *NF1* co-alterations were present in 16–23% of clinical cases and enriched post-TKI treatment (5) (*Table 1*).

D. Wnt Signaling pathway

The β-catenin protein, encoded by the *CTNNB1* gene, represents a key component of the WNT signaling pathway, functioning as a nuclear transcriptional activator for target genes regulating cellular proliferation and differentiation (103). When signaling through the WNT pathway is down-regulated, β-catenin is degraded by the adenomatous polyposis coli (APC)/actin/glycogen synthase kinase 3β (GSK-3β) destruction complex, whereas aberrant β-catenin activating mutations prevent GSK-3β-mediated phosphorylation and degradation, keeping β-catenin active (57).

CTNNB1 activating co-mutations show consistent co-selection with EGFR mutations in patient tumors or ctDNA (*Table 1*). *CTNNB1* activating mutations are enriched in late stage *EGFR*-mutated NSCLC patients (5–10% of patients) (5,39), supporting a potential role for WNT pathway activation in promoting tumor invasion and metastasis (4,5,16,57-60). One study, for example, showed that overexpression of CTNNB1 S37F in preclinical models enhanced tumor cell invasion and reduced sensitivity to first generation EGFR TKIs through inhibition of apoptosis (5).

E. Transcription factors

MYC

MYC amplification was first reported in lung cancer in 1983 and has been correlated with primary resistance to EGFR TKI treatment as well as decreased PFS and OS (104-106) (*Table 1*). The MYC protein acts as a regulator of cell cycle progression and cellular transformation through its activity as a transcriptional activator (107). Concurrent *MYC* and *EGFR* alterations were observed in ~10% NSCLC patients with a trend toward enrichment in patients progressing after TKI therapy (5). Combinatorial treatments of EGFR and MYC inhibitors are currently being tested in preclinical EGFR-mutant lung cancer models as a potential approach to overcome primary TKI resistance (61).

NKX2-1

NKX2-1, also known as TTF-1, is a homeodomain transcription factor with an essential role in peripheral lung development (108). Specifically, abundant NKX2-1 expression was associated with EGFR-mutant LUADs in which NKX2-1 exerts a lineage-survival oncogenic role (62,63). Moreover, *NKX2-1* gene amplification was detected in 15% and 11% of EGFR-mutant NSCLC patients at baseline and after TKI progression, respectively (16) (*Table 1*). Yamaguchi *et al.* demonstrated that NKX2-1 trans-activates ROR1 receptor which binds to EGFR favoring PI3K-AKT signaling as well as phosphorylates SRC, further enhancing AKT activity (109).

F. Gene fusions

Oncogenic fusions have been detected in 3–10% of EGFR-mutant LUADs at resistance to osimertinib treatment (23) (*Table 1*). The potential oncogenic fusion proteins described include: *FGFR3-TACC3*, *RET-ERC1*, *CCDC6-RET*, *NTRK1-TPM3*, *NCOA4-RET*, *GOPC-ROS1*, *AGK-BRAF*, *ESYT2-BRAF*, *SPTBN1-ALK*, *PLEKHA7-ALK*, and *EML4-ALK* (17,23,34,47,64-67). Of note, combination of osimertinib and a RET inhibitor, in presence of *CCDC6-RET* fusion, or crizotinib, in presence of *EML4-ALK* fusion, were effective therapies for overcoming resistance in case studies (65,67).

G. Cell cycle-G1/S regulators

The RB1 protein acts as a tumor suppressor, inhibiting G1/S progression through the cell-cycle (110). Its activity is regulated by CCND1-CDK4/6 complex-dependent phosphorylation, which tags RB1 for degradation and activates E2F transcription factors which mediates G1/S entrance (110). Inactivating *RB1* mutations occur in ~10% of EGFR-mutant NSCLC, mostly co-occurring with *TP53* mutations (5,16,88,111,112) (*Table 1*). EGFR-mutant LUAD carrying inactivated *RB1*, *TP53* genes have higher probability of small cell transformation following EGFR TKI (112-114).

Additional frequent concurrent G1/S gene alterations detected in patients progressing to first- and second-line osimertinib are: *CDK4* (7–10%), *CDK6* (7%), *CCNE1* (7%) amplifications and *CDKN2A* (25%), *CDKN2B* (20%) deletions, with *CDKN2A/2B* deletions being typically clonal alterations (5,16,88) (*Table 1*). Importantly, *CDK4/6* amplifications correlated with resistance to first generation EGFR TKIs and reduced PFS to osimertinib (5). Preclinical studies using a combination of 1st or 2nd generation EGFR TKIs and a CDK4/6 inhibitor showed a significant delay in the onset of EGFR TKI resistance when compared to EGFR TKI monotherapy (115,116). Accordingly, clinical testing with combinatorial treatments of EGFR and CDK4/6 inhibitors are ongoing (117).

Therapeutic implications and perspectives

Intrinsic and acquired resistance to single agent EGFR-targeted therapies remains a significant challenge in the treatment of lung cancer. The impact of co-occurring genomic alterations on clinical outcomes for EGFR-mutant NSCLC patients is becoming more appreciated. Numerous EGFR-dependent and independent co-alterations are associated with reduced EGFR TKI sensitivity and shorter PFS. Additionally, tumor mutation burden increases in post-TKI treatment samples, adding complexity to the heterogeneous genomic landscape.

Specifically, advanced *EGFR*-mutated NSCLCs undergo dynamic modulation of tumor sub-clonal populations, with multiple pathogenic alterations co-existing in pre- and post-TKI specimens (*Figure 1*) (5). Overcoming this dynamic and complex process will require an equally adaptive treatment approach based on real-time monitoring of tumor genomic complexity. Computational frameworks that profile patterns of dynamic, actionable alterations in real-time and identify optimal therapy switching strategies have been described in preclinical models of co-occurring *EGFR*, *BRAF*, and *MET* alterations (*Figure 1*) (119). The implementation of this strategy in patients could rely on: (I) frequent liquid biopsy and profiling of ctDNA to detect emerging genomic alterations during therapy and (II) mathematical modeling to identify therapy strategies that pre-empt or overcome the outgrowth of resistant tumor subclones (*Figure 1*) (5,119).

Using broad sequencing panels, it is now possible to characterize the intra-tumor heterogeneity and identify multiple co-existing, actionable oncogenic targets within individual tumors (118). Moreover, a genomic pathway-centric analysis of broad-panel next generation sequencing data has been recently proposed to identify patterns of therapeutic vulnerabilities in LUAD patients (118). The use of up-front, targeted therapy combinations and dynamic therapy switching could thus represent more durable treatments in advanced stage NSCLC and are currently investigated in pre-clinical and clinical trials (117,119).

Overall, systematic data collection and management, novel computational tools, high throughput pre-clinical testing, real-time tumor genomic assessments, and flexible

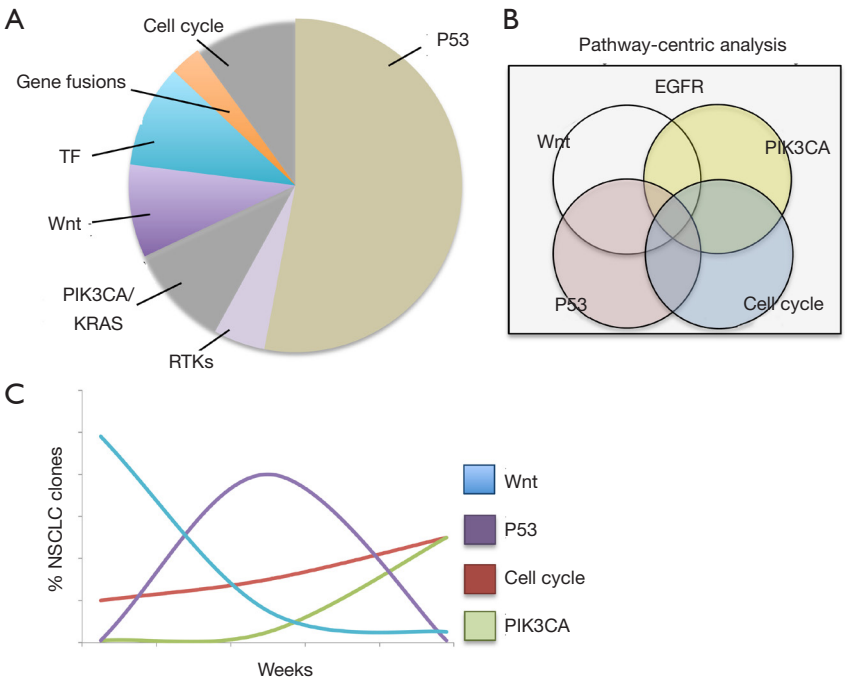

Figure 1 Classification of concurrent genomic alterations in EGFR-mutated NSCLC. (A) Most frequently co-occurring pathways altered in EGFR-mutated NSCLC are highlighted in the pie chart (not necessarily mutually exclusive), which can impact the efficacy of EGFR-targeted therapy. (B) A pathway-centric classification of tumor genomic co-alterations could guide the selection of combination therapy approaches (118). (C) Real-time monitoring of tumor clonal evolution through liquid biopsies in conjunction with mathematical modeling may inform combination therapy treatment schedules and switching strategies (hypothetical clonal evolution shown) (119). EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; TF, transcription factors.

clinical trial designs will be required to more effectively employ precision therapeutic approaches that address the genomic complexity and heterogeneity present in EGFR-mutant NSCLC (*Figure 1*).

Acknowledgments

Funding: The Damon Runyon Cancer Research Foundation P0528804 (to CM Blakely), Doris Duke Charitable Foundation grant # 2018110 (to CM Blakely), and V Foundation V2018-020 (to CM Blakely).

Footnote

Provenance and Peer Review: This article was commissioned by the Guest Editor (Trever G. Bivona) for the series "Mechanisms of resistance to EGFR-targeted therapy" published in *Journal of Thoracic Disease*. The article was sent for external peer review organized by the Guest Editor and the editorial office.

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/jtd.2020.03.78). The series "Mechanisms of Resistance to EGFR-targeted Therapy" was commissioned by the editorial office without any funding or sponsorship. CMB reports grants from Astrazeneca, grants from Takeda, grants from Novartis, grants from Mirati, grants from Spectrum, grants from Roche, personal fees from Revolution Medicines, personal fees from Foundation Medicine, outside the submitted work. The other authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Cite this article as: Gini B, Thomas N, Blakely CM. Impact of concurrent genomic alterations in epidermal growth factor receptor (*EGFR*)-mutated lung cancer. J Thorac Dis 2020;12(5):2883-2895. doi: 10.21037/jtd.2020.03.78