

Review Article

EGFR-TKI resistance in NSCLC patients: mechanisms and strategies

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Abstract: The epidermal growth factor receptor (EGFR) is a kind of receptor tyrosine kinase (RTK) that plays a critical role in the initiation and development of malignant tumors via modulating downstream signaling pathways. In non-small cell lung cancer (NSCLC), the activating mutations located in the tyrosine kinase domains of EGFR have been demonstrated in multiple researches as the “Achilles’ heel” of this deadly disease since they could be well-targeted by epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs). However, it’s still too early to celebrate since the first-generation EGFR-TKIs such as gefitinib and erlotinib have only achieved limited clinical benefits and acquired resistance to this kind of drugs occurred inevitably in almost all the NSCLC patients. In order to make the most of EGFR-TKIs and develop more effective regimens for the NSCLC patients, researchers majoring in different aspects start a battle against EGFR-TKI resistance. Challenging as it is, we still progress stably and step firmly toward the final victory. This review will summarize the major mechanisms of acquired resistance to EGFR-TKIs, and then discuss the development of rationally designed molecular target drugs in accordance with each mechanism, in the hope of shedding light on the great achievements we have obtained and tough obstacles we have to overcome in the battle against this deadly disease.

Keywords: Non-small cell lung cancer, epithelial growth factor receptor, tyrosine kinase inhibitors, drug resistance, molecular targeted therapy

Introduction

Cancers of the lung have long been the leading cause of cancer-related death all over the world. According to the statistic announced by the American Cancer Society, in the year 2014, the United States is projected to witness 224,210 new cases of lung cancer, ranking the second frequent cancer in both male and female. 159,260 cases are estimated to finally die of lung cancer, accounting for 27.2% of all the cancer-related death [1]. Non-small cell lung cancer (NSCLC), the major object of this review, is the largest subgroup of lung cancer, occurring at the frequency of about 80% [2]. Generally speaking, the therapeutic effect of NSCLC is far from satisfactory. Although great progress has been made in the chemotherapy, almost no more than 10 months median overall survival (OS) can be achieved even by the most effective platinum-based chemotherapeutic regimens [3].

EGFR and EGFR-TKIs in lung cancer

With deeper digging into the molecular events underlying the oncogenesis and progression of NSCLC, epidermal growth factor receptor (EGFR), a kind of tyrosine kinase receptor, became one of the landmark targets of NSCLC therapy. It is a member of the HER family, which also includes HER2 (ErbB2), HER3 (ErbB3), HER4 (ErbB4) [4]. When EGFR’s extracellular domain binds to its ligand, such as epidermal growth factor (EGF) and transforming growth factor- α (TGF- α), it forms dimers with other EGFR or other HER family members to get itself auto-phosphorylated at the key tyrosine residues. Subsequently, the phosphorylated EGFR further activates several downstream signaling pathways such as PI3K/AKT/mTOR, RAS/RAF/MAPK, JAK/STAT, which play the critical roles in regulating multiple cellular processes, including proliferation, survival and apoptosis [4, 5]. The constitutive activation of EGFR signaling path-

way, caused by gene mutations or by gene amplification or both, has been demonstrated to have close connection with the initiation, progression and poor prognosis of NSCLC [6]. EGFR activating mutations, majorly located in the tyrosine kinase domains and in the form of a base-pair deletion at exon 19 (delE746_A750, account for about 54%) or a point mutation at exon 21 (L858R, account for about 43%), occur at about 20% of NSCLC patients, with significantly increased proportions in the subgroup of adenocarcinoma histology (40%), female sex (42%), Asian ethnicity (30%), and never-smoker status (51%) [7]. They enable the EGFR to activate, without the ligand binding, the downstream molecules [8]. As such, the tumor cells are consequently addicted to the EGFR signal pathway. The first-generation EGFR tyrosine kinase inhibitors (EGFR-TKIs), gefitinib and erlotinib, designed to reversibly compete for the ATP binding sites and thus block EGFR-induced downstream signaling activation, have and are being extensively investigated in NSCLC treatment. The landmark Iressa Pan-Asia Study (IPASS) showed gefitinib could significantly prolong median progression free survival (PFS) compared with carboplatin/paclitaxel in the subgroup of patients with EGFR mutation-positive tumors (median PFS 9.5 months versus 6.3 months; hazard ratio [HR] 0.48, 95% CI 0.36 to 0.64, $p < 0.001$), together with much improved qualification of life (QoL) and delayed deterioration of symptoms [9, 10]. Similar benefits of EGFR-TKIs in EGFR mutation-positive NSCLC patients were shown in several other large-scale studies, such as WJTOG3405 comparing gefitinib with cisplatin/docetaxel as first-line treatment in Asian patients (median PFS 9.2 months versus 6.3 months; HR 0.489, 95% CI 0.336 to 0.710, $p < 0.0001$), OPTIMAL comparing erlotinib with chemotherapy as first-line treatment in Asian patients (median PFS 13.1 months versus 4.6 months; HR 0.16, 95% CI 0.10 to 0.26, $p < 0.0001$), EURTAC comparing erlotinib with standard chemotherapy as first-line treatment in European patients (median PFS 9.7 months versus 5.2 months; HR 0.37, 95% CI 0.25 to 0.54, $p < 0.0001$) [11-13]. However, the superiority of gefitinib in patients with high *EGFR*-gene-copy number over docetaxel was not proven in the INTEREST study [14]. In addition, the carboplatin/paclitaxel combination demonstrated superior efficiency than gefitinib in the *EGFR*-gene-amplified

but *EGFR* mutation-negative subgroup (median PFS: 5.5 months versus 1.5 months; HR 2.85, 95% CI 2.05 to 3.98, $p < 0.001$) [9]. Therefore, it is plausible that it's the *EGFR* mutation status that determines the tumor responses to EGFR-TKIs. This conclusion is widely recognized and dramatically promotes the clinical practices of detecting *EGFR* mutation status and applying the EGFR-TKIs to the subset of *EGFR* mutant patients. However, problems arose that almost all the patients with initial dramatic responses to gefitinib or erlotinib ultimately underwent tumor progression and inevitably became resistant to them mostly within 6-12 months, which has been defined as "acquired resistance" [15, 16]. Several mechanisms underlying have been discovered, but more arduous efforts should be made since 30% of the required resistant cases remain unexplainable. In addition, a better understanding of the mechanisms is only the first step and coping rationally with them is the critical next step to win the battle against EGFR-TKI resistance.

Mechanisms of EGFR-TKI resistance in NSCLC

In a long period of time, we had no idea about the mechanisms leading to the EGFR-TKI resistance until 2005, when Susumu Kobayashi and his colleagues firstly discovered the T790M mutation after sequencing the *EGFR* gene of a patient with acquired resistance to gefitinib [17]. After that, a series of tremendous successes have been achieved in this field (Figure 1).

Gatekeeper mutation in EGFR: T790M mutation

T790, located in the ATP binding pocket, is named "the gatekeeper residue" as it determines the affinity of ATP-competitive EGFR-TK inhibitors to EGFR-TK. Substitution of Threonine 790 with Methionine (T790M) increases the ATP's affinity to EGFR and attenuates the binding efficacy of gefitinib and erlotinib consequently [18]. Approximately 50% of the acquired resistance developed to erlotinib or gefitinib is linked to T790M mutation and the proportion could be underestimated as more accurate prevalence of 68% was achieved using LNA-PCR/sequencing assay [19]. While the reason remains enigmatic, the good news is that the patients with T790M at the time of TKI failure tend to have longer post-progression survival (PPS) than those without such a mutation [20].

EGFR-TKI resistance in NSCLC

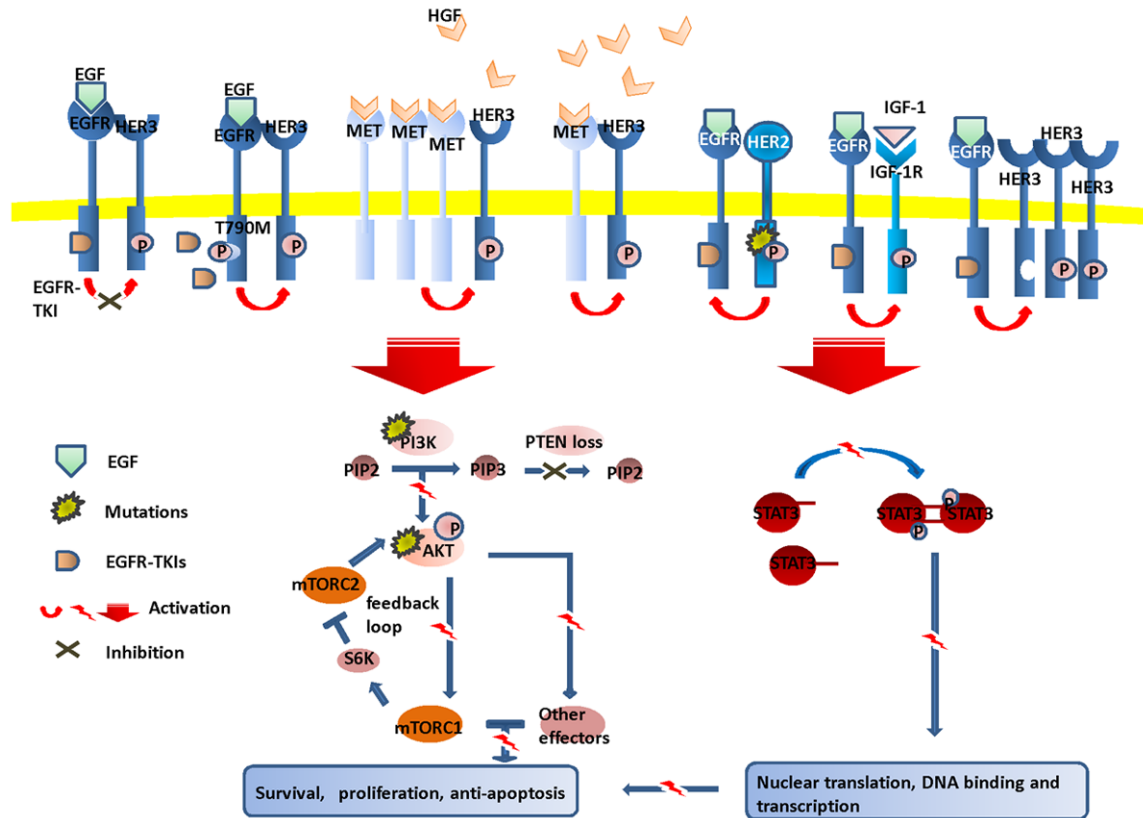


Figure 1. Various mechanisms of EGFR-TKI resistance. Despite of constitutive existence of first-generation of EGFR-TKIs, the tumor cells manage to survive and proliferate via EGFR-T790M mutation, upregulation of MET/HGF, HER2 mutations, overexpression of HER3, persistent activation of IGF-1R, mutations of PIK3CA/AKT, loss or downregulation of PTEN and abnormal dimerization of STAT3.

Compensatory contribution of other RTKs

c-MET: MET receptor, a trans-membrane tyrosine kinase encoded by proto-oncogene *MET*, has been highlighted as an important cause for acquired resistance of NSCLC to gefitinib or erlotinib. As the ligand for MET receptor, hepatocyte growth factor (HGF, also known as scatter factor), once binding to MET receptor, will promote the phosphorylation of MET tyrosine kinase and subsequently trigger the activation of downstream PI3K/AKT/mTOR pathway, which is the key signaling pathway for cell proliferation, survival and anti-apoptosis [21, 22]. A lot of preclinical trials have shown that uncontrolled activation of MET was oncogenic and facilitated the cells to become malignant, invasive, metastatic and EGFR-TKI resistant. Mechanisms underlying are multiple, such as MET and HGF overexpression, *MET* gene amplification or mutation [23, 24]. The *MET* gene amplification emerges as one of the most relevant mechanisms, and is correlated with poor

clinical outcomes [25]. About 22% of the EGFR-TKI acquired resistant specimens have been demonstrated to possess *MET* gene amplification [26, 27]. Of note, the MET over-activation in most circumstances occurs via increased transcription and expression of MET protein instead of *MET* amplification [28]. In addition, over 20 mutations have been identified in *MET* and the majority of them were found to be germline mutations [29]. They are oncogenic in a large variety of human cancers, including NSCLC. The most frequent mutations locate in the semaphorin domain (affecting HGF binding), the juxtamembrane domain (affecting the actin cytoskeleton, cell motility and migration) and the TK domain (activating MET even in the absence of HGF) [30]. The occurrence rates of *MET* gene mutations vary along ethnic and racial lines, with the highest frequency occurring in East Asian [29]. Generally speaking, the *MET* gene mutation in NSCLC patients was less frequently reported (8~13%) and their correspondence with acquired EGFR-TKI resistance

EGFR-TKI resistance in NSCLC

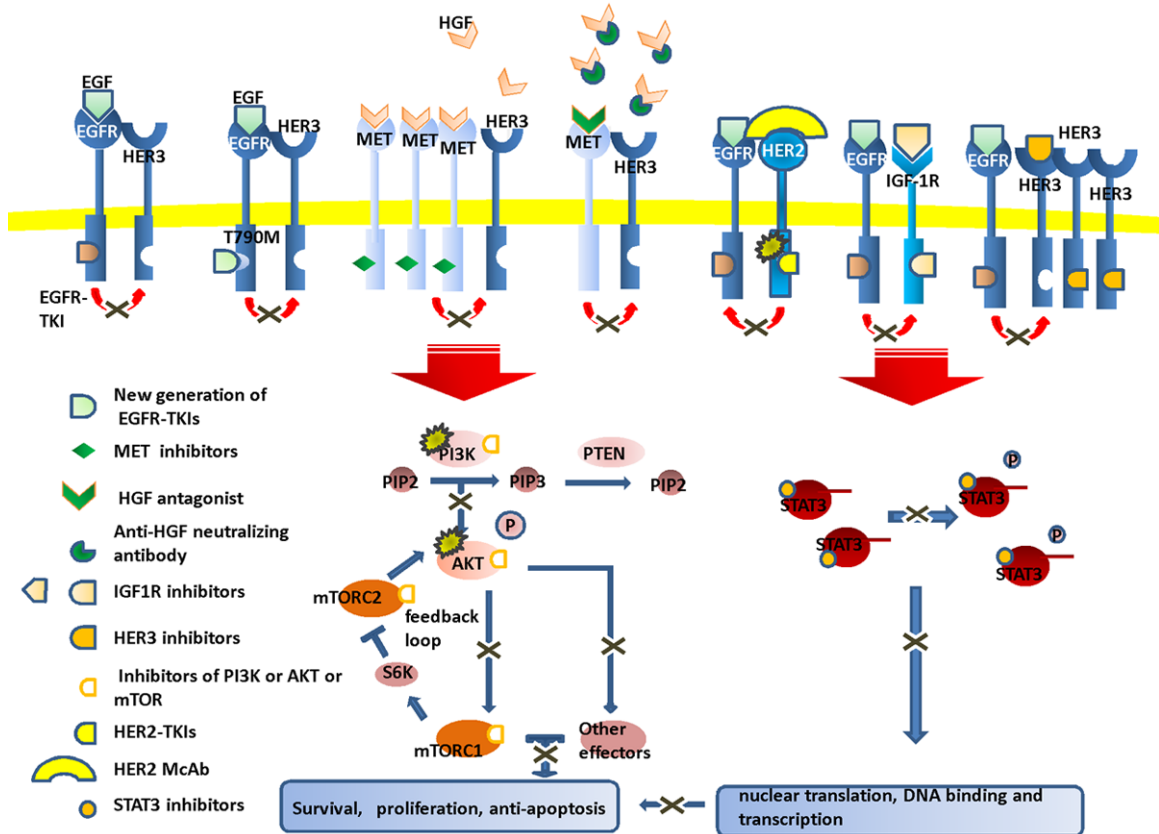


Figure 2. New agents to overcome EGFR-TKI resistance. To cope with the molecular events responsible for the resistance, multiple targeted agents are developed including the new-generation EGFR-TKIs, MET/HGF inhibitors, HER2 inhibitors, HER3 inhibitors, IGF-1R inhibitors, PI3KCA/AKT inhibitors and STAT3 inhibitors.

needs to be interpreted with cautions [31]. Moreover, HGF overexpression is a much more frequent event in acquired resistant NSCLC tumors (61% in a Japanese research) than in the sensitive ones (10%), supporting the role for HGF overexpression in promoting drug resistance [32]. The high-level of HGF can be secreted by lung cancer cells themselves and stromal fibroblasts in the tumor microenvironment as well [33]. How HGF contributes to the resistance formation can be elucidated by two known mechanisms. Firstly, HGF can stimulate the activation of PI3K signal pathway by means of binding and activating MET receptor without the involvement of EGFR, which attenuates the addition of tumor cells at EGFR signal pathway and thus eliminates the anti-tumor potency of EGFR-TKIs [23]. Secondly, the presence of HGF can induce a tyrosine kinase-independent function of EGFR, which is interacting with other tumor relevant proteins such as CDCP1, AXL, EohE2 et al. [34]. Taken together, the aberrant activation of HGF-MET pathway is one of the

main obstacles to fight against EGFR-TKI resistance in NSCLC and greater efforts are warrant to make steps further in this aspect.

IGF-1 Receptor: Growing evidences have emerged for the involvement of the IGF-1 Receptor (IGF-1R) pathway in the acquisition of resistance to EGFR-TKIs. Constitutive activation of IGF-1R pathway has been detected in multiple gefitinib or erlotinib resistant lung cancer lines. This connection was further proven by testing primary NSCLC samples using immunohistochemistry (IHC) that higher IGF-1R expression level was detected in acquired gefitinib resistant patients than those who were sensitive [35]. The way IGF-1R interfering the anti-tumor activity of EGFR-TKIs seems to be complicated. Amandine Hurbin et al. suggested IGF-1R managed it by inhibiting apoptosis via amphiregulin [36]. Besides, Floriana Morgillo and his colleagues also found that IGF-1R could be activated via forming a heterodimer with EGFR after erlotinib treatment. The activated

EGFR-TKI resistance in NSCLC

Table 1. Summary of the targeted agents and clinical trials to overcome resistance to EGFR-TKI

Target	classification	Agents	Clinical trials finished	Clinical trials ongoing
T790M	Covalent EGFR-TKI inhibitor	Afatinib	LUX-lung 1 [78], LUX-lung 2 [163], LUX-lung 3 [80], LUX-lung 4 [79], LUX-lung 6 [81]	NCT01085136, NCT01466660, NCT01523587
	T790M-selective inhibitor	CO-1686, PKC412, AZD9291	Phase I studies of PKC412 [89, 164]	NCT01526928, NCT02147990 (TIGER-2), NCT01802632 (AURA), NCT02094261 (AURA2)
HGF	HGF antagonist	NK4		
	Anti-HGF antibody	TAK-701, ficlatuzumab	Phase I study of TAK 701 [94] Phase II trial comparing gefitinib with and without ficlatuzumab [165]	NCT01039948
MET	MET tyrosine kinase inhibitors	Tivantinib, cabozantinib, INC280	Phase II study comparing erlotinib with or without tivantinib [97] Phase III study comparing erlotinib with and without tivantinib [29]	NCT01580735, NCT02049060, NCT01377376, NCT00596648, NCT01866410, NCT01639508, NCT01610336
	MET mono-antibody	Onartuzumab	phase II trial comparing erlotinib with and without onartuzumab [166]	NCT01456325 (MetLung) NCT01887886, NCT02031744
HER3	Anti-HER3 agents	Pertuzumab	Phase II study exploring pertuzumab plus erlotinib [107]	
HER2	Anti-HER2 agents	Lapatinib, trastuzumab, neratinib	Phase II trial evaluating lapatinib as monotherapy [167] Phase II trial evaluating neratinib [168]	NCT01827267, NCT01184482, NCT01306045, NCT00004883, NCT00758134, NCT01827267, NCT00266877
IGF-1R	IGF-1R mono-antibody	R1507	Phase II study exploring erlotinib plus placebo or R1507 [169]	NCT00773383
PI3K	PI3K inhibitors	BAY 80-6946, LY294002		NCT01460537, NCT01411410, NCT01404390, NCT00962611
mTOR	mTORC1 inhibitors	Everolimus, temsirolimus, sirolimus	Phase II exploring everolimus plus gefitinib [111] Phase II study of temsirolimus monotherapy [170]	NCT00079235, NCT01827267, NCT01737502, NCT01050985, NCT01482156
	Dual PI3K/mTOR inhibitors	BEZ235, PF-04691502, PKI-402	Phase I study of PF-04691502 monotherapy [122]	NCT01482156, NCT00620594, NCT01343498, NCT01508104
AKT	AKT inhibitors	MK-2206, Enzastaurin,	Phase I trial exploring MK-2206 plus carboplatin/paclitaxel, docetaxel or erlotinib [124] Phase I trials exploring enzastaurin plus gemcitabine/cisplatinin [126] Phase II study, exploring enzastaurin plus cisplatin/pemetrexed [127]	NCT01147211, NCT01294306, NCT00452413
HSP90	Hsp90 inhibitors	Ganetespi, AUY-922, DS-2248, retaspimycin, 17-DMAG	Phase II study evaluating IPI-504 monotherapy [140] Phase II study evaluating ganetespi monotherapy [141]	NCT01348126, NCT01031225, NCT01259089, NCT01784640, NCT01288430, NCT01362400, NCT01427946

Table 2. Summary of miRNAs involved in EGFR-TKI resistance in NSCLC

miRNA name	Expression in resistant NSCLC	Targets	References
miR-21	Up	PDCD4, MDR1, PTEN and bcl-2	[171-173]
miR-103 and -203	Down	PKC-ε, SRC, Dicer.	[174, 175]
miR-126	Down	PIK3R2, CRK, VEGF	[176-178]
miR-128b	Down	EGFR	[179]
miR-214	Up	PTEN, p38, MAPK	[180, 181]
miR-221, miR-222	Up	PTEN, APAF-1, BIM, TIMP3	[174, 182]
MiR-145	Down	c-MYC, AKT, ERK, OCT4	[183-185]

IGF-1R keeps transmitting extracellular survival signals to downstream mediators such as PI3K/AKT and MAPK to stimulate mammalian target of rapamycin (mTOR), which mediates the synthesis of EGFR and anti-apoptotic survivin proteins [37]. How IGF-1R signaling is activated in TKI resistant cells remains largely unknown. Evidences arose that it was at least partially induced by the down-regulation of major IGF carrier protein IGFBP3 [38]. Co-treatment of IGF-1R inhibitors such as α-IR3, AG1024, R1507 with EGF-TKIs enhanced TKI-induced growth inhibition and apoptosis, offering a potential approach to overcome the primary resistance of EGFR-TKIs in NSCLC [39, 40].

HER2: *HER2* mutation occurred at about 2% of patients with NSCLC, significantly more frequent in never smokers, adenocarcinoma histology, oriental ethnicity and female gender [41]. Almost all *HER2* mutations locate in exon 20, encoding the kinase domain of *HER2* protein [42]. Distinct from the other family members, *HER2* has strong kinase activity but has no identified ligand binding domain. Thus, it has to form heterodimers with the other family members including activated EGFR to get trans-phosphorylated [43]. The dependence of *HER2* activation on the trans-phosphorylation of EGFR determines the strong inhibition of EGFR-TKIs on the wide type *HER2*. However, when *HER2* mutates in the kinase domain, it becomes EGFR-independent and in turn trans-phosphorylates EGFR even in the presence of EGFR-TKIs [44]. Consequently, NSCLC cells holding the mutant *HER2* are more potent in activating downstream signal transducers and exert resistance to EGFR-TKIs and knockdown of the mutant *HER2* succeed in restoring sensitivity to EGFR-TKIs. Moreover, EGFR-TKI resistance can also be induced by *HER2* gene amplification or protein overexpression [45]. Therefore, it pro-

vides a rationale to detect the *HER2* status and add *HER2*-targeted agents such as lapatinib, trastuzumab and dacomitinib to EGFR-TKIs in the treatment of certain NSCLC patients [46-48].

HER3: While EGFR and *HER2* have been

ranked as two of the most heated targets in NSCLC targeting treatment, it was not rare that even effective blockage of EGFR and *HER2* in EGFR-driven or *HER2*-driven xenografts could not durably suppress AKT signaling. Growing evidences have shown the involvement of *HER3*, another *HER* family member, in this phenomenon [49]. The persistent activation of *HER3* was detected in human cancer cells and essential for the binding activated EGFR, *HER2*, MET to PI3K/AKT signaling. *HER3* lies upstream the PI3K signaling pathway and functions as an accessory partner of EGFR and *HER2* [50]. When *HER3* forms a dimer with EGFR or *HER2*, it gets phosphorylated by intrinsic tyrosine kinase activity of EGFR or *HER2* and then couples them to the PI3K signaling pathway since EGFR and *HER2* lack the tyrosine-phosphorylated Tyr-X-X-Met motif necessary for docking PI3K while *HER3* contains 7 copies [49, 51]. *HER3* is dependent on EGFR and *HER2* to get tyrosine-phosphorylated, and meanwhile, EGFR and *HER2* have to rely on *HER3* to recruit and trans-phosphorylate the PI3K molecular. Ideally, EGFR-TKIs can disassociate the EGFR-*HER3* dimers and consequently block the downstream PI3K/AKT pathway. However, continuous EGFR-TKI exposure often triggers the overexpression of *HER3* as a result of the loss of AKT-mediated negative feedback signaling. The overexpressed *HER3* promotes the forward shift in the equilibrium of the *HER3* phosphorylation-dephosphorylation reactions and results in superphosphorylated state of *HER3* and AKT, which requires much higher concentration of RTK-TKIs to fully disassociate the heterodimers or much more potent *HER3*-targeted drugs to completely dephosphorylate *HER3* [52].

Activation of compensatory signaling pathways

PI3K/AKT/mTOR signaling pathway: Just as mentioned before, the PI3K/AKT/mTOR signal-

ing pathway downstream the RTKs (EGFR, MET, HER2, HER3) plays a key role in promoting the proliferation, survival, drug resistance of cancer cells [53]. When an extracellular ligand binding to the receptor (such as EGF binding to EGFR) leads to phosphorylation and activation of the RTK, PI3K is triggered to catalyze the phosphorylation of phosphatidylinositol biphosphate (PIP2) into phosphatidylinositol triphosphate (PIP3). Accumulation of PIP3 localizes AKT to the plasma membrane, where AKT gets phosphorylated by 3-phosphoinositide-dependent kinase 1 (PDK-1) indirectly or by PIP3 directly. The activated AKT regulates the phosphorylation of downstream effectors and thus leads to changes in gene expression and cell behavior. The major downstream effector is the mammalian target of rapamycin complex 1 (mTORC1), which regulates its downstream molecules such as the eIF4E-binding proteins (4E-BPs) and S6 kinases (S6K1 and S6K2) [54]. PI3K pathway regulation appears to be quite complicated. It's negatively regulated by an important tumor suppressor, phosphatase and tensin homolog (PTEN), which converts active PIP3 to inactive PIP2, thus inactivating PI3K/AKT signaling. In addition, S6K can attenuate PI3K signaling via the S6K-IRS1 feedback loop and AKT phosphorylation via inhibiting mammalian target of rapamycin complex 2 (mTORC2) which is necessary for full AKT activation together with PIP3 [55]. Therefore, it's rational to raise the hypothesis that there is a negative connection between the activation of this pathway and the susceptibility to EGFR-TKIs. Indeed, AKT activation and mTOR phosphorylation were frequently present in NSCLC patients (43-90% and 60-90%, respectively) [56]. They can be induced by multiple mechanisms, including *AKT* gene mutation, mutations and amplifications of *PIK3CA* (the gene encoding the main catalytic subunit of PI3K) as well as loss or reduced expression of PTEN [57-59]. Although the clinical data about the prevalence of PI3K/AKT/mTOR pathway induced EGFR-TKIs resistance is rare, the involvement of PI3K/AKT/mTOR pathway induced EGFR-TKIs resistance was repeatedly confirmed by preclinical researches. For instance, Jeffery A. Engelman et al. transfected a NSCLC cell line with P110 α E545K, a *PIK3CA* oncogenic mutation, resulting in dramatically suppressed sensitivity to gefitinib compared with the control ones [60]. Horimasa Takeda et al. reported a 5-fold

increased IC50 value of gefitinib in NSCLC cells when PTEN was knocked-down using a vector containing short heparin RNA against PTEN [61]. Reversely, reconstitution of PTEN can restore the tumor cell killing potency of TKIs [59].

JAK2/STAT3 pathway: The STAT (Signal Transducer and Activator of Transcription) protein, especially the STAT3, was reported to be another critical downstream signal transducer of activated EGFR besides the RAS/RAF/MAPK and PI3K/ARK [62]. Inappropriate activation of STAT3 was observed at high frequency (50%) in lung cancer patients [63]. Activated STAT3 acts as a transcriptional factor transferred into the nucleus and regulating the transcription of target-genes which mediate survival (survivin, bcl-xl, mcl-1, cellular FLICE-like inhibitory protein), proliferation (c-fos, c-myc, cyclin D1), invasion (matrix metalloproteinase-2), and angiogenesis (vascular endothelial growth factor) [64]. The activation of STAT3 is a complex event. It begins when the extracellular proteins such as cytokines (IL-6, interferon), growth factors (EGF, PDGF) bind to the corresponding receptors. The ligand bound receptor recruits and phosphorylates the tyrosine kinases JAK2 (JAK family: JAK1, JAK2, JAK3 and Tyk2. JAK2 is most frequently involved in oncogenesis), which eventually leads to STAT3 protein phosphorylation, dimerization and activation. In addition, Several other kinases can activate STAT3 directly without the mediation of JAK, such as the Src family (Lck, Src), Abl family (BCR-Abl), EGFR, IGF-1R, protein kinase C (PKC) and so on [65]. Apart from the remarkable role of JAK2/STAT3 in oncogenesis, clues have emerged recently that aberrant JAK2/STAT3 activation was partially involved in attenuated sensitivity of NSCLC to the first generation EGFR-TKIs. In an erlotinib resistant NSCLC cell line, the phosphorylation of STAT3 was remarkably increased although EGFR and MAPK were markedly suppressed by the existing erlotinib [66]. When adding the JAK inhibitor JSI-124 together with erlotinib, the sensitivity to erlotinib was restored both in vitro and in vivo [67]. However, abundance of relevant questions remain unrecognized and warrant for further investigation, such as the mechanisms underlying the aberrant JAK2/STAT3 activation, the safety and anti-tumor potency of JAK or STATs inhibitors,

how to make the best of EGFR-TKIs via drugs-combination and so on.

SCLC phenotypic transforming: Although little is known about the phenotypic transforming of NSCLC histology to small cell lung cancer (SCLC), increasing attention has been drawn to this phenomenon. Lecia V. Sequist and her colleagues recently reported 5 in 37 (14%) NSCLC patients were diagnosed of SCLC after developing resistance to EGFR-TKIs. This SCLC transforming was confirmed with positive immunohistochemical staining in the post-resistant biopsy specimen for synaptophysin which specially exists in the SCLC [68]. Besides, genetic analysis showed the newly-emerged SCLCs harbored the same EGFR mutations with the original NSCLC ones, and this was surprising since it was an exceedingly rare event for SCLC to harbor EGFR mutations (about 4%) [68, 69]. Actually, the SCLC transforming from NSCLC has been previously reported as a series of individual cases [70-72]. Those cases shared the same characteristics of never-smoking, female, transforming from adenocarcinoma to SCLC after developing TKI resistance. No exact mechanism underlying this phenomenon has been launched. Probably, SCLC cells originate from the minor pre-existent cells under the selection pressure of EGFR-TKIs, or trans-differentiate from the adenocarcinoma cells, or arise from the multi-potent stem cells [72]. Analyzing the genetic and phenotypic biopsy specimens all along the course of NSCLC diagnosis and treatment can help us for the better understanding this drug resistant mechanism, and more importantly, help us to make wiser clinical decisions throughout the course of the disease [68].

EMT phenotypic transforming: Epithelial to mesenchymal transition (EMT) refers to a complex program by which close-connected and polar-ranged epithelial cells turn into spindle-shape mesenchymal cells with significantly increased motility, invasiveness, and resistance to apoptosis [73]. Loss of epithelial cell junction proteins such as E-cadherin and the gain of mesenchymal markers such as vimentin or fibronectin are the distinctive molecular events that are frequently accompanied with EMT [74]. Increasing evidences have emerged to correlate EMT with increased resistance of NSCLC cells to EGFR-TKIs, and oppositely, the

NSCLC cells with lower degree of EMT shown sensitivity to EGFR-TKIs even without EGFR activating mutations both in vitro and in xenografts [75, 76]. Clinically, EMT has been proven to contribute about 5% to EGFR-TKI resistance via biopsy paired specimens achieved pre- and post-resistance [68]. However, how EMT promotes TKI resistance remains unknown. Nevertheless, some common mechanisms such as EGFR T790M mutation or MET amplification are unlikely the culprit.

Strategies to reverse TKI resistance

Knowledge of mechanisms underlying the drug resistance keeps updating and this consequently stimulates the development of diverse new drugs and combinational regimens to overcome EGFR-TKIs resistance. For the next part, we will focus on those newly emerged drugs and regimens in terms of their action modes as well as their anti-tumor potency both in vivo and in vitro (**Figure 2** and **Table 1**).

Targeting EGFR T790M

Secondary generation EGFR-TKIs: In view of the fact that resistant tumor cells are still addicted to the EGFR signaling pathway, new drugs which can irreversibly block EGFR-TK via the formation of covalent bonds in the pocket of the catalytic site should be able to increase the potency of EGFR-TK inhibition. One such inhibitor, the second generation EGFR-TKI afatinib (BIBW-2992), designed to bind covalently with Cys-797 at the gatekeeper pocket, can potently and selectively block both wild-type and mutant forms of ErbB family receptors (EGFR, HER2, ErbB3 and ErbB4) [18]. It has been confirmed of a sustained and potential antineoplastic activity in a battery of cell lines and xenograft models. Compared with erlotinib, gefitinib or lapatinib, it showed 100-fold more sufficient ability to inhibit the enzymatic activity of the L858R-T790M EGFR and comparable potency against HER2 [77]. Therefore, a series of clinical trials have been conducted to evaluate clinical benefits of afatinib. LUX-lung1 explored afatinib versus placebo in NSCLC patients who failed in prior treatment with at least 1 line of platinum-based chemotherapy and disease progressed after at least 12 weeks of treatment of erlotinib or gefitinib. Among them, 83% were EGFR-mutant positive, including those progressed after a short complete response

(CR)/partial response (PR) to prior EGFR-TKIs and/or were treated for ≥ 48 weeks with prior EGFR-TKIs. Although the primary endpoint overall survival (OS) showed no benefit from afatinib (afatinib versus placebo: 10.8 months versus 12.0 months; HR 1.08, 95% CI 0.86-1.35, $p=0.74$), the afatinib group experienced a prolonged progress-free survival (PFS) (3.3 months versus 1.1 months; HR 0.38, 95% CI 0.31-0.48; $p<0.0001$), better confirmed objective response rate (ORR) (7% versus $<1\%$), better disease control rate for ≥ 8 weeks (58% versus 18%) and improved life quality in the overall population than that of placebo. Especially in the subgroup of patients meeting the Jackman criteria of acquired resistance to EGFR-TKI, afatinib showed a pronounced PFS benefit over placebo (4.5 months versus 1.0 months) [78]. This encouraging result was further confirmed by LUX-lung 4, which was conducted in patients failed prior erlotinib and/or gefitinib [79]. Furthermore, first-line afatinib has been compared with standard combination chemotherapeutic regimens in LUX-lung 3 (afatinib versus combination chemotherapy of cisplatin and pemetrexed) and LUX-lung 6 (afatinib versus combination chemotherapy of cisplatin and gemcitabine) [80, 81]. The pooled analysis of those two large open-label phase III studies announced in the 2014 ASCO Annual Meeting proved the favorable anti-tumor activity of afatinib. Median OS was prolonged from 24.3 months in chemotherapy group to 27.3 months in the afatinib group (HR=0.81; CI 0.66 to 0.99; $p=0.037$). Especially in the Del19 subgroup, the HR was 0.59 (CI 0.45 to 0.77; $p<0.001$), preferable for the afatinib group [82]. Besides, LUX-lung 7 and LUX-lung 8 directly comparing afatinib with gefitinib or erlotinib are still recruiting patients.

Despite of the benefits on the T790M-positive NSCLC patients achieved from the utility of second generation EGFR-TKIs, the improvement seems to be rather limited. It is largely caused by the insufficient drug concentrations as the toxicity of this drug limits the blood concentrations under the level required to overcome the EGFR T790M mutation [83]. In the clinical trial of LUX-lung 1, adverse events such as skin rash, acne and diarrhea were observed with at least 10% higher incidence than the placebo group, including 150 in 390 (38%) patients needed a dose reduction and 70 (18%) patients discontinued afatinib because of those adverse

events [78]. The mechanism underlying goes to the potent blockage of afatinib against both the wild-type EGFR and the mutant ones [77].

Third generation of EGFR-TKIs: In view of this, the third generation EGFR-TKIs that selectively target the mutant EGFR, in particular the T790M mutation, but exhibiting minimal potency toward the wild-type receptor emerged in quick succession. CO-1686 is one of them and exhibits potent inhibition of EGFR T790M but circumvents wild-type EGFR [84]. The phase I/II clinical trial evaluating the safety, pharmacokinetic and preliminary efficacy in patients with previously treated EGFR mutant NSCLC of CO-1686 has been half done (NCT01526928). Some promising discoveries have been released in the 4th European lung cancer conference (ELCC) in 2014. It reported overall response rate (ORR) of 80%, PFS of over 6 months in T790M-positive NSCLC patients who administered CO-1686 following the development of resistance to erlotinib. Another phase II study exploring CO-1686 as a second-line therapy in the T790M mutant patients who failed the previous EGFR-TKI treatment, TIGER-2, just started recruiting patients this year (NCT-02147990). WZ4002 is another mutation-selective EGFR inhibitor that displays high degree of selectivity against EGFR T790M even at low concentrations, and no significant inhibition of wild-type EGFR was accompanied in pre-clinical studies [85]. Several studies are further evaluating its anti-EGFR T790M activity and one of them showed pronounced anti-tumor capacity and tolerable toxicity in NSCLC bearing mice when combined with MET inhibitor crizotinib [86]. However, no study assessing WZ4002 in human beings is available yet. Midostaurin (PKC412), the multi-target tyrosine kinase inhibitor (targeting PKC, FLT3, AKT, c-kit, PDGFR et al.), is deeply studied and widely utilized to treat patients with AML or MDS [87]. It has been proven in T790M mutation positive NSCLC cell lines and tumor models of a novel function of potent and selective inhibition of EGFR T790M other than FLT3. Meanwhile, it showed no significant blockage of wide-type EGFR activity which means much less toxicity and better tolerance of PKC412 versus first- and second-generation TKIs [83]. Clinical evaluation of PKC412 as a PKC, FLT3 inhibitor against AML and MDS is under investigation. No obviously toxicity against normal cells was

observed despite of its multi-targeting capacity [88]. A phase I trial conducted in 23 patients with advanced NSCLC showed good effectiveness of PKC412 at the dosage of 50 mg/day combined with gemcitabine and cisplatin and no significant side reactions including myelosuppression were observed [89]. However, further studies are warranted to assess its efficacy in overcoming T790m induced TKI resistance.

Targeting HGF-MET pathway

HGF antagonist: NK4, composed of the N-terminal hairpin and amino-terminal four kringle domains of hepatocyte growth factor (HGF), acts as the competitive antagonist of HGF as well as an angiogenesis inhibitor against VEGFR and FGF. The two functions of NK4 are mutually independent but interactional with each other, making it potential in suppressing malignant tumors in both tumor growth and spreading [90, 91]. NK4 over-expressed in the established lung cancer xenograft models dramatically suppressed the tumor growth, angiogenesis, and metastases without obvious side effects [92]. However, the relevance of NK4 to overcome TKI resistance is not yet investigated.

Anti-HGF neutralizing antibody: TAK-701 is a potent humanized monoclonal antibody to HGF. It works by suppressing the HGF binding to MET receptor and thus restrains the proliferation effects of MET pathway. Wataru Okamoto et al. treated the HGF overexpression induced TKI-resistant NSCLC cells with TAK-701 and gefitinib and observed significant suppression on the activation of MET, ERK, AKT and cell growth [93]. This promising outcome indicated that the addition of TAK-701 to gefitinib was a feasible strategy to abrogate EGFR-TKI resistance induced by HGF. NCT00831896 is the first clinical trial to determine the safety, tolerability, and pharmacokinetics profile of TAK-701 in adult patients with advanced non-hematologic malignancies. It showed good tolerance at the dosage of up to 20 mg/kg every other week and preferable negative conversion ratio of free HGF (71.4%) [94].

MET tyrosine kinase inhibitors: Tivantinib is a non-ATP-competitive small molecule MET inhibitor. It works by stabilizing the inactive conformation of MET, and thus hinders the activation

of downstream signaling pathway. Given the well-tolerance and potential ability of tivantinib both as single-agent therapy and in combination with erlotinib announced in several preclinical and phase I clinical trials, a series of work have and are being carried out to evaluate its antitumor efficacy [95, 96]. An international randomized phase II study conducted on 167 NSCLC patients suggested benefits from the combination of tivantinib and erlotinib, compared with erlotinib alone (PFS 3.8 months versus 2.3 months; adjusted HR 0.68, 95% CI 0.47-0.98, $p=0.04$). Especially in the patients of non-squamous histology, a subgroup with higher possibility to be MET-positive (75% in non-squamous histology versus 12% in squamous histology), a significant prolongation has been demonstrated in median PFS (4.4 months versus 2.3 months, adjusted HR 0.61, 95% CI 0.47-0.98, $p<0.05$) and median OS (9.9 months versus 6.8 months; adjusted HR 0.58, 95% CI 0.34-0.99, $p<0.05$) [97, 98]. Based on this encouraging result, a phase III randomized study of erlotinib plus tivantinib in pretreated but TKI-naive advanced non-squamous NSCLC patients was carried out [98]. Unfortunately, this trial was recommended to cease since the interim analysis shown the achieved PFS benefit could not carry over into OS prolongation [29]. Anyway, a variety of clinical trials alike are underway, including a phase II single-arm study (NCT01580735) investigating tivantinib plus erlotinib in EGFR-TKI resistant locally advanced or metastatic NSCLC subjects and a phase III randomized placebo-controlled study (NCT01377376) exploring tivantinib plus erlotinib versus erlotinib monotherapy in advanced and EGFR mutation negative non-squamous NSCLC. Cabozantinib (XL-184) is a novel tyrosine kinase inhibitor that is under evaluation in preclinical and clinical studies. The characteristics of inhibiting MET and VEGFR-2 make it the powerful drug in inhibiting tumor growth and survival, as well as blood vessel formation, invasiveness and metastasis [99]. It also displays inhibitory activity against several other RTKs including RET, KIT, FLT3 and TEK, which are all critical molecules in oncogenesis [100]. Several studies preliminarily explored the potential of cabozantinib combining EGFR-TKI in gefitinib- or erlotinib-resistant NSCLC cell lines in vitro and xenograft tumors in vivo. The outcomes were quite encouraging since extensive tumor shrinkage and decreased tumor invasiveness

and metastasis were observed by this combination regimen [99, 100]. The results of a phase I/II randomized clinical trial (NCT00596648) assessing cabozantinib, either alone or in combination with erlotinib in acquired resistant NSCLC patients are expected in the near future. Recently, a novel MET inhibitor, INC280 caught our attention. In the 2014 ASCO Annual Meeting, Yi-Long Wu et al. disclosed the latest phase Ib results of the ongoing phase Ib/II study exploring INC280 plus gefitinib in NSCLC patients who were EGFR mutated, MET-amplified and failed in the prior EGFR inhibitor treatment. 6 in 41 (15%) evaluable patients obtained partial responses and all those responders had high MET status [101]. The phase II trial to further verify the efficiency of this combination regimen is currently ongoing (NCT01610336).

MET monoclonal antibody: Onartuzumab (MetMab) is a newly developed humanized monoclonal antibody targeting MET. It blocks the HGF binding to MET, and thus attenuates the activation of its downstream transducers and effectors [102]. It was evaluated in a randomized phase II trial comparing erlotinib with and without onartuzumab in advanced NSCLC patients. Despite of no statistically significant differences between the two arms in the overall population, the combinational arm showed 47% reduction in the risk of disease progression and significant prolongation in median PFS (2.9 months versus 1.5 months; HR 0.53, 95% CI 0.283-0.99, $p=0.04$) and median OS (12.6 months versus 3.8 months; HR 0.37, 95% CI 0.19-0.72, $p=0.002$) in the subset of MET-positive, which was confirmed to be associated with bad prognosis. In contrast, MET-negative patients experienced earlier progression in the combinational arm (median PFS loss of 1.3 months; HR 1.82, 95% CI 0.99-3.32, $p=0.05$) and shorter survival (median OS loss of 7.2 months; HR 1.78, 95% CI 0.79 to 3.99, $p=0.16$), indicating its potential to reverse or prevent MET induced TKI resistance [103]. Unfortunately, the inspiring outcome could not carry over to the phase III study (MetLung, NCT01456325), which enrolled 499 MET-positive advanced NSCLC patients. It was recommended by an independent data review committee to cease since no improved OS (6.8 months versus 9.1 months; HR 1.27, $p=0.068$),

PFS (2.7 months versus 2.6 months; HR 0.99, $p=0.92$), and overall response rate (8.4% versus 9.6%; $p=0.63$) was observed in the onartuzumab plus erlotinib arm [104].

Targeting HER3 pathway

Anti-HER3 agents: Several measures can overcome acquired resistance induced by HER3 activation. One strategy is to increase the dosage of EGFR-TKIs to fully inactivating concentration or develop more potent anti-HER drugs since studies have convicted TKI-refractory HER3 phosphorylation relays on unsuppressed HER2 [52]. Another strategy is to develop anti-HER3 agents blocking HER3 activation, such as antibodies against HER3 [105]. Pertuzumab is a novel HER2/HER3 dimerization inhibitor [106]. The outcome of a phase II study exploring the combination of pertuzumab and erlotinib in 41 relapsed NSCLC patients was published recently. In contrast to the original intention of greater activity as a result of a broader HER family blockage, this combination regimen shown only modest anti-tumor potency but generally poor tolerance [107]. Thus, until more powerful anti-HER drugs are developed, multi-targeting treatment by combining EGFR-TKI with inhibitors on other targets such as mTOR or PI3K is still the first choice [49].

Targeting PI3K/AKT/mTOR pathway

PI3K inhibitors: BAY 80-6946, developed as an intravenous drug, can inhibit pan-class I PI3K with high potency and selectivity. Sustained response was observed in animal models bearing patient-derived NSCLC xenografts when co-treated with BAY 80-6946 and paclitaxel [108]. Till now, at least 4 phase I studies are further evaluating the clinical value of BAY 80-6946 in multiple advanced tumors including NSCLC (NCT01460537, NCT01411410, NCT01404390, NCT00962611).

mTORC1 inhibitors: Rapamycin and its analogues are the most developed inhibitors targeting PI3K/AKT/mTOR pathway. Everolimus (RAD001), an orally bioavailable rapamycin derivative, is a potent inhibitor of mTORC1. It showed promising efficacy in restoring sensitivity of gefitinib resistant NSCLC cell lines which harbor PIK3CA mutation or lose PTEN [109, 110]. Clinical studies have been carried out to assess the efficacy of combining everolimus

with EGFR-TKIs or chemotherapies. In contrast to the promising outcomes of preclinical trials, the efficacy of those combination regimens in NSCLC patients seem rather modest. A phase II combination study conducted by Katharine A. Price et al in EGFR-TKI naive patients with advanced NSCLC only witnessed response rate of 13% (8 of 62 patients), which did not meet the prespecified response threshold to pursue further study of combining everolimus and gefitinib [111]. Another phase I study carried out by Jean-Charles Soria et al showed better disease-control rate (DCR), CR or PR rate, median duration of stable disease in the group of advanced NSCLC patients received with the combination of everolimus and erlotinib (50%, 12%, 9.3 months) as second- or third-line therapy than in patients with erlotinib alone (45%, 8.9%, 7.9 months) [112]. This result suggests that the combination treatment with erlotinib and everolimus may be superior to treatment with erlotinib alone in the molecularly unselected population. Many trials on various combinations are ongoing, such as everolimus plus cetuximab, everolimus plus capecitabine and everolimus plus cisplatin, in the hope of finding more effective treatment combinations [113-115].

Of note, the success achieved by rapamycin and its analogues, such as everolimus appears to be limited, and many preclinical trials have noticed the abnormal AKT phosphorylation even in the existence of those agents [116]. This phenomenon can be explained by their nature of merely inhibiting mTORC1, which leaves AKT constitutive activated via the abrogation of S6K-IRS1-PI3K feedback loop or via mTORC2 [55, 116]. Thus, it's rational to develop new agents that target both mTORC1 and mTORC2, or novel regimens of combining different kinds of PI3K/AKT/mTOR inhibitors to maximize pathway inactivation and overcome TKI resistance.

Dual PI3K /mTORC1 /mTORC2 inhibitors: The inhibitors co-targeting PI3K/mTORC1/mTORC2 were designed on the basis of high-level structural homology of the catalytic site of PI3K and mTOR. Their nature of dual inhibition makes them superior than mere mTORC1 inhibitors by attenuating PI3K/mTORC2 induced AKT activation. They strongly reduced the proliferation rate as well as inducing a dramatic apoptotic response [117]. Although the dual PI3K/

mTORC1/mTORC2 targeting compounds are still in early development, pronounced anti-tumor capacity have been observed in many preclinical researches. BEZ235, a novel ATP-competitive PI3K/mTOR dual inhibitor, has displayed striking anti-proliferative effects in HGF induced EGFR resistant lung cancer cell lines and a xenograft model even as monotherapy [118]. Furthermore, when BEZ235 was combined with everolimus, marked synergy was achieved in the inhibition of NSCLC cell growth both in vitro and in vivo [119]. BEZ235 is under investigation in phase I/II trials either by itself alone or in combination with other agents in patients with various types of cancer including NSCLC (NCT01482156, NCT00620594, NCT01343498, NCT01508104 et al). Since inhibition of mTOR signaling can induce autophagy to enable cell survival under unfavorable conditions, combining autophagy-blocking drugs may enhance the anti-tumor capacity of BEZ235 [120]. PF-04691502 is another dual PI3K and mTOR inhibitor. It has entered phase I clinical trials as it pronouncedly reduced AKT phosphorylation in PTEN null or PIK3CA mutated cells, and induced tumor shrinking in NSCLC xenografts resistant to first-generation EGFR-TKI [121]. According to the phase I clinical study in patients with advanced solid tumors, oral administration of PF-04691502 reduced phosphorylated AKT and STAT3, although no objective responses were observed [122].

AKT inhibitors: AKT is the critical downstream effector of PI3K. Aberrant phosphorylation of AKT signaling results in reduced sensitivity of NSCLC to receptor TKIs. MK-2206, an oral AKT inhibitor, is undergoing varies of in vitro and in vivo studies. The co-treatment with MK-2206 and erlotinib has shown a synergistic effect in erlotinib-insensitive NSCLC cell lines and tumors [123]. The combination effects could be explained by the blockage of both AKT and ERK pathways. On this background, a phase I trial was carried out to evaluate the effect of MK-2206 plus carboplatin and paclitaxel, docetaxel or erlotinib on patients with advanced solid tumors including 13 NSCLC patients. Well-tolerance was observed as well as early evidence of antitumor activity. One NSCLC patient treated with MK-2206 and docetaxel after 2 prior lines of platinum-based chemotherapy and erlotinib treatment obtained additional partial response (PR) [124]. Based on those

promising outcomes, following trials are underway to further investigate the combination of MK-2206 with other standard cytotoxic or targeted treatments (NCT01147211, NCT01294306). Enzastaurin is initially developed as an anti-tumor agent via anti-angiogenesis activity. But later it was proven to directly suppress tumor cell proliferation and induce tumor cell death owing to the newly discovered potential of blocking AKT pathway [125]. Enzastaurin has been investigated in a phase I clinical trials in combination with gemcitabine and cisplatin in patients with advanced solid tumors. This regimen was well-tolerated and resulted in clinical benefits in 16 out of 33 patients [126]. In a phase II randomized study, the combining regimen of cisplatin/pemetrexed plus enzastaurin was evaluated in patients with advanced NSCLC. Despite of the pleasant outcome of 7 PR and 2 SD in 13 patients in the lead-in phase, this clinical trial was suspended due to the negative result of the other two peer phase II studies [127].

Inhibiting epithelial-mesenchymal transition

Histone deacetylase (HDAC) inhibitors: Since the loss of E-cadherin is the specific event of EMT, and has been associated with poor clinical outcome as well as bad response to EGFR-TKIs in NSCLC, attempts have been made to restore the E-cadherin expression using histone deacetylase (HDAC) inhibitors. Entinostat (MS-275) is one of such drugs. It succeeded in restoring E-cadherin expression, growth-inhibitory and apoptosis-promoting effects of erlotinib and gefitinib preclinically [128]. Furthermore, a randomized phase II trial evaluating the combination of erlotinib with entinostat, a kind of oral HDAC inhibitor, in advanced NSCLC patients who failed in prior chemotherapy have completed with acceptable adverse events and prolonged OS (9.4 months versus 5.4 months; HR 0.3, 95% CI 0.13 to 0.92, $p=0.03$) as well as PFS (3.68 months versus 1.88 months; HR 0.55, 95% CI 0.22 to 1.37, $p=0.19$) in the subset of patients with high E-cadherin levels [74]. Certainly, HDAC inhibitors can activate the expression of many silenced tumor suppressors, leading to tumor inhibition independent of EMT reversion [129]. In addition, the molecular events underlying EMT are so complicated that more breakthroughs are needed to develop EMT-targeting drugs overcoming TKI-resistance.

Newly emerged multi-targeted agents

HSP90 inhibitors: The heat shock protein 90 (Hsp90) is a kind of molecular chaperones responsible for the conformational maturation and stabilization of its substrate proteins [130]. Elevated levels of Hsp90 have been demonstrated in NSCLCs and they protected the tumor cells against unfavorable conditions via stabilizing proteins necessary for tumor survival [131]. Multiple well-known oncogenetic drivers and drug-resistance causing proteins of NSCLC including EGFR, HER2, MET and AKT are the substrates of Hsp90 [132, 133]. When one of them is blocked, the tumor cells are smart enough to signal through the alternative kinases via oncogenic switching frequently induced by Hsp90. Furthermore, the mutated or overexpressed forms of those oncogenetic proteins preferentially are more dependent on Hsp90 to remain stable than their wild-type counterparts [133]. All mentioned above suggest the increasing virtue of Hsp90 inhibitors as multi-targeted agents in the treatment of oncogene addicted NSCLC [134]. Multiple preclinical NSCLC models containing one or more mutant oncogenes have been established in vitro and in vivo. Following the exposure to HSP 90 inhibitors, the expression of these oncogenetic proteins were compromised and cell growth was abrogated [135-137]. Several newly-developed Hsp90 inhibitors such as STA-9090 (Ganetespib), AUY-922, DS-2248, IPI-504 (retaspimycin) and 17-DMAG are in active clinical evaluation either as monotherapy or in combination with other agents (NCT01348126, NCT01031225, NCT01259089, NCT01784640, NCT01288430, NCT01362400, NCT01427946 et al) [138-141].

Targeting miRNAs: miRNAs are a class of 18-24 nt small noncoding RNAs that negatively regulate the target genes expression either by inhibiting mRNA translation or by promoting mRNA degradation [142]. Emerging evidences have suggested their master regulatory roles in oncogenesis either as oncogenes or as tumor suppressor genes [143]. In NSCLC, upregulated miRNA30b, miRNA30c, miRNA221, miRNA222 are associated with resistance to gefitinib treatment through the regulation of PTEN and ARAF-1 expression, while miRNA103 and miRNA203 induce apoptosis in gefitinib resistant cells and promote mesenchymal to epithelial transformation via the down-regulation of

PKC- ϵ , SRC and Dicer [144]. Since miRNA expression in gefitinib and erlotinib resistant NSCLC cell lines were profiled, various relevance of miRNAs to TKI resistance have been gradually clarified (Table 2). Particularly, miRNAs typically target a cluster of genes rather than one specific gene, modulating miRNAs by introduction of suppressive miRNAs or inhibition of oncogenetic miRNAs might bring a powerful therapeutic strategy to overcome EGFR-TKIs resistance [143]. This idea is not inconceivable since studies in vivo have succeeded in delivering anti-sense oligonucleotides against targeted miRNAs into laboratory animals with up-regulated levels of corresponding miRNAs [145, 146]. In addition, new technologies able to raise the suppressive miRNA levels in tumor cells via introducing synthetic miRNA mimics keep arising one after another [147]. Besides, oncogenetic miRNAs and suppressor miRNAs can be either down-regulated or up-regulated by a much safer approach using several “natural agents” such as isoflavone, 3,3'-diinodolymethane (DIM) [148]. Although several key issues of this approach remains unsolved such as the delivery methods, stability and safety in humans, miRNA-based treatment will finally take their place in the battle against EGFR-TKIs resistance.

Conclusions and perspectives

EGFR activating mutations have been overoptimistically recognized as the Achilles' heel of NSCLC after some clinical successes have been achieved. Unfortunately, almost all patients initially responding to gefitinib or erlotinib would inevitably progress to develop acquired resistance. To our relief, the constantly updating knowledge of the mechanisms underlying has already been translated to the development of novel targeted agents, rational combination regimens and improved survival benefits to NSCLC patients.

In order to make use of these molecular tools to full extend, many hurdles lying ahead must be overcome. Firstly, there remains the requirement of exploring unknown mechanisms of resistance since nearly 30% of the acquired resistance cannot be explained by the mechanisms recognized. New potential mechanisms are keeping proposed such as CRKL gene amplification, AXL kinase over-activation, acqui-

sition of stem cell-like properties, loss of an EGFR-amplified chromosome 7 [149-154]. Certainly, they need to be confirmed in clinical researches with large sample sizes before the development of novel drugs targeting these synthetically lethal vulnerabilities. Secondly, too many failures in the battle against NSCLC have told us that the tumors are much smarter than we thought. They outsmart single-target drugs to escape from the fate of death by various mechanisms. And it is not rare to see several different resistant mechanisms existing in one patient synchronously or continuously. In regard of that, the exploration of potential and well-tolerated combinational regimens and novel “one target multiple” drugs should be the hotspots of future drug development. Meanwhile, the profiles of different EGFR-TKI resistant subgroups classified by different mechanisms should be described to provide individualized anti-tumor regimens such as combination of various drugs targeting different targets. Thirdly, biopsy specimen for the genetic profiles of cancer patients should be performed all along the course of the disease since resistant clones can emerge up to 10 months before radiological changes [155]. An early discovery of new genetic aberrations in NSCLC patients such as T790M mutation, *PI3KCA* mutation or *MET* amplification makes it possible for patients to cease expensive and toxic EGFR-TKIs treatment regimens and start potentially successful changes in accordance to real-time genetic status as soon as possible. The traditional tissue biopsy is not always accessible, and only one third patients enrolled in the IPASS trial were available for EGFR-mutation status examination [10]. In addition, single biopsy is sometimes impossible to represent heterogeneous landscape of the tumor [156]. “Liquid biopsy” screening for circulating tumor DNA (ctDNA) within a blood sample drawn from NSCLC patients has emerged and is under active evolution to reflect tumor genetics and tumor dynamics [156-158]. T790M mutation, *KRAS* or *BRAF* mutations, *PIK3CA* mutation, *HER2* gene amplification have already been successfully detected in NSCLC, colorectal cancer or breast cancer patients by this technology [159-162]. The information of all tumor sites provided by the “Liquid biopsy” will surely better monitor the EGFR-targeted therapies although many critical obstacles are lying ahead. Nevertheless, the battle against

acquired resistance to EGFR-TKI in EGFR-mutant NSCLC has come a long way, although there is still a long way ahead.

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Disclosure of conflict of interest

We have no conflict of interest to claim.

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