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Epidermal growth factor receptor tyrosine-kinase inhibitor treatment resistance in non-small cell lung cancer: biological basis and therapeutic strategies

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Abstract Lung cancer remains the leading cause of cancer-related death. Non-small cell lung cancer (NSCLC) represents 85 % of all lung cancer cases and it is classified into three major subtypes: adenocarcinoma, squamous cell carcinoma and large-cell carcinoma. In the past years, molecular-targeted therapies have been developed in order to improve response, survival and quality of life in patients with advanced NSCLC. Lung cancers harboring mutations in the epidermal growth factor receptor (EGFR) respond to EGFR tyrosine-kinase inhibitors (TKIs). However, virtually all patients with initial response relapse due to acquired resistance. Better understanding the biology of these tumors and mechanisms of EGFR TKIs resistance could shed some light on research of new therapeutic options in this setting. This review aims to emphasize on EGFR involved lung cancer pathway, primary and acquired mechanisms of TKIs resistance, and discuss agents currently used in clinical development in this emerging scenario.

Keywords Non-small cell lung cancer · EGFR · TKI · Overcome resistance · Reversible–irreversible TKI · First-generation–second-generation TKI

Abbreviations

NSCLC	Non-small cell lung cancer
SCLC	Small cell lung carcinoma
EGFR	Epidermal growth factor receptor
VEGF	Vascular endothelial growth factor
TKI	Tyrosine-kinase inhibitor
RTKs	Tyrosine-kinase receptor family
PI3K	Phosphatidylinositol 3-kinase
WT	Wild-type
KRAS	Kirsten rat sarcoma viral oncogene homolog
PTEN	Phosphatase and tensin homologue
MET	Mesenchymal–epithelial transition factor
EMT	Epithelial to mesenchymal transition
RECIST	Response Evaluation Criteria in Solid Tumors
WHO	World Health Organization

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Introduction: understanding the biological basis

EGFR family encloses a group of 4 members: ERBB1 (EGFR-HER1), ERBB2 (Neu or HER2), ERBB3 (HER3) and ERBB4 (HER4). EGFR belongs to tyrosine-kinase receptor family (RTKs); each of these proteins possesses three domains: the extracellular domain, involved in recognizing and binding the ligands; the transmembrane domain, involved in interactions between receptors; and the intracellular domain, with intrinsic tyrosine-kinase activity [1].

This receptor family recognizes different related growth factors [2], such as epidermal growth factor (EGF), transforming growth factor alpha (TGFα), heparin-binding EGF-like growth factor (HBEGF), amphiregulin, betacellulin, epiregulin and neuregulins [3]. Receptor-ligand interaction induces a conformational change which produces a receptor homo or heterodimerization, with a subsequent phosphorylation of tyrosine residues on different intracellular coupled proteins and of the receptor itself.

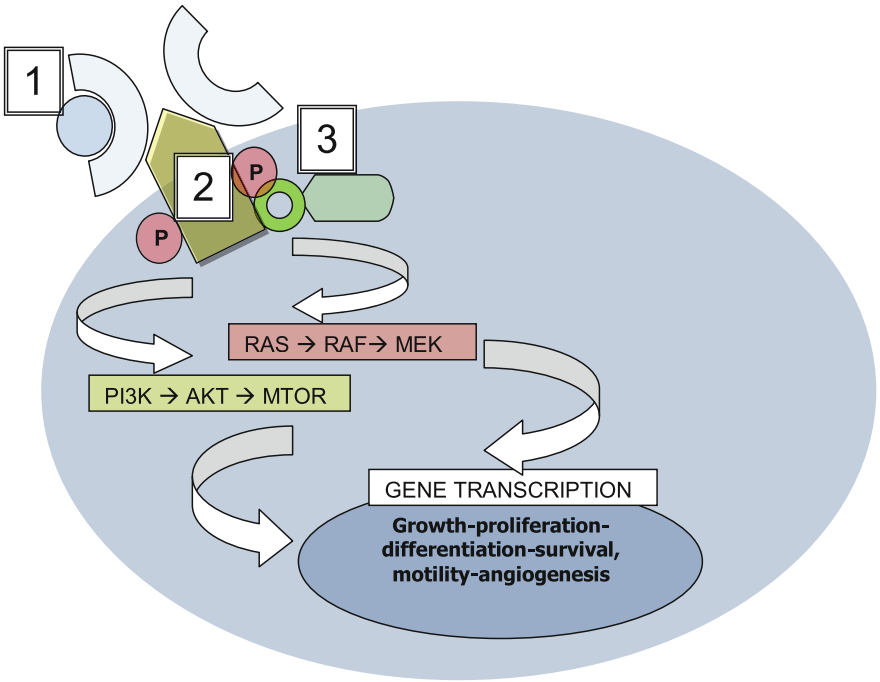

Fig. 1 EGFR pathway and TKI mechanism of action in cell with EGFR-sensitizing mutation. *1* Ligand binding to extracellular portion of RTKs leads to receptor dimerization and phosphorylation of tyrosine residues located at the intracytoplasmatic enzymatic domain. This activates downstream pathways involved in many functions of the cell. *2* EGFR mutations in ATP cleft of the tyrosine-kinase domain generate stabilization in the interaction with ATP, stimulating phosphorylation of tyrosine residues with a hyperactivation of cellular downstream pathways, even in absence of ligands. *3* ERLOTINIB and GEFITINIB inhibit the phosphorylation and tyrosine-kinase activity of the intracellular ATP-binding domain of EGFR through a competitive mechanism

This process leads to activation of intracellular signal transduction pathways, such as phosphatidylinositol 3-kinase PI3K/AKT/mTOR and the RAS/RAF/Mitogen-activated protein kinase (MAPK)/ERK kinase (MEK)/extracellular-signal-regulated kinase (ERK) pathways [4].

These pathways are involved in cell transformation and tumor progression:

(a) PI3K is a family of proteins involved in the regulation of cell growth, metabolism, proliferation, glucose homeostasis and vesicle trafficking. A direct antagonist of PI3K is the phosphatase and tensin homologue (PTEN) which directly reverses the activity PI3K by dephosphorylating phosphatidylinositol 3,4,5-triphosphate (PIP3) into phosphatidylinositol 4,5-bisphosphate (PIP2), and therefore plays an important role as a negative controlling element of incoming signals. The loss and/or mutation of PTEN in various cancers lead to hyperactive PI3K pathway. Under phosphorylation of mediated proteins, the prosurvival AKT kinase is recruited. Likewise, AKT represents one of the main regulators of mTORc1 (mammalian target of rapamycin complex 1), a complex involved in protein translation, ribosome construction and autophagy [5].
(b) The RAS/RAF/MEK/ERK pathway plays an essential role in cell proliferation, differentiation and survival. Activated RAS recruits RAF family members (BRAF). RAF stimulates MEK, a kinase that phosphorylates tyrosine/threonine residues of MAPK, allowing its activation and the modulation of different cellular process, such as growth, proliferation, differentiation, survival, motility and angiogenesis [6].

In physiological conditions, ligand binding to extracellular portion of RTKs leads to receptor dimerization and phosphorylation of tyrosine residues located at the intracytoplasmatic enzymatic domain; this activates downstream pathways involved in many functions of the cell [7]. But inappropriate mechanism of RTKs activation might lead to activation of cellular transduction, even in absence of ligand. Mutation in TK domain of this receptor generates hyperactivation of cellular downstream pathways signaling, a key role for cell proliferation and tumorigenesis [8] (Fig. 1).

EGFR and lung cancer: the paradigm of targeted therapy

Different mechanisms activate EGFR signaling in lung cancers. EGFR is overexpressed, assessed by immunohistochemistry (IHC), in more than 60 % of lung cancers [9].

In some cases, genomic analyses show the amplification of chromosomal region 7p12, where the EGFR gene is located [10]. NSCLC cells can release EGF ligands. These ligands may induce juxtacrine, autocrine, paracrine, and/or endocrine signaling [11]. Another important mechanism of RTK activity disruption is a mutation that affects the activity of tyrosine-kinase domain [12]. Thus, NSCLC cells that depend on EGFR for survival constitutively activate the receptor through a combination of genetic mutations, overexpression of EGFR and their ligands.

In virtue of the above, pharmacological selective block of EGFR has evolved as a treatment paradigm in NSCLCs patients with mutations in EGFR TK domain. Actually, gefitinib [13] and erlotinib [14] represent the two major first-generation TKIs approved for advanced NSCLC.

Mutational analysis of the entire EGFR coding sequence of gefitinib-responsive tumors was performed in 2004 [15]. The identification of specific somatic-sensitizing mutations within the tyrosine-kinase domain of EGFR and its correlation with encouraging responses in this subgroup of patients marked a turning point in lung cancer targeted therapy. These mutations are allocated near the ATP cleft of the tyrosine-kinase domain, generating stabilization in the interaction with ATP, stimulating phosphorylation of tyrosine residues and causing intracellular transduction activation in an aberrant manner [12].

Erlotinib and gefitinib inhibit the phosphorylation and tyrosine-kinase activity of the intracellular ATP-binding domain of EGFR through a competitive mechanism. Thus, the inhibition of the receptor achieves a down-regulation of its related intracellular pathways [16]. From a chemical perspective, gefitinib and erlotinib are effective in EGFR-mutant NSCLC because they are more potent inhibitors of EGFR mutants than of the wild-type (WT) EGFR kinase. In fact, inhibition of WT EGFR in the normal tissues contributes to the dose-limiting toxicity of EGFR TKIs.

In the ISEL phase III trial [17], gefitinib was compared to placebo in second-line setting, and it did not demonstrate any overall survival (OS) gain. Subgroups of never smokers and Asians achieved better median OS with gefitinib and some patients developed amazing tumor responses with gefitinib [18]. BR21 trial [19] showed an OS improvement with erlotinib vs. placebo in second- and third-line setting. Response rate (RR) was 8.9 % for erlotinib arm and <1 % for placebo arm. OS for the erlotinib group was 6.7 months compared with 4.7 months for placebo arm (HR 0.7, $p < 0.001$). Exploratory multivariate analyses showed that Asian origin, adenocarcinomas histology and no smoking history were significantly independent predictors of survival.

The identification of somatic mutations of EGFR has led to the development of numerous trials. IPASS (Iressa Pan-Asia Study) phase III trial [20] compared for the very first time EGFR TKIs (gefitinib) with a chemotherapy doublet (carboplatin/paclitaxel) in a first-line setting of advanced NSCLC. Superiority of gefitinib in terms of progression-free survival (PFS) was reported (HR 0.74, 95 % CI 0.65–0.85; $p < 0.001$). Subgroups' analysis showed that patients with EGFR mutations had higher overall response rate (ORR) with gefitinib compared with chemotherapy (71.2 vs. 47.3 %, $p = 0.0001$); a significant difference in PFS favoring gefitinib in this subgroup was reported (9.5 vs. 6.3 months, HR 0.48; $p < 0.001$). In the subgroup of patients with EGFR mutation negative tumors, ORR with gefitinib was 1.1 %, and PFS was significantly longer with chemotherapy (HR 2.85, 95 % CI 2.05–3.98; $p < 0.001$). Higher ORR in EGFR mutation-positive patients, who received chemotherapy (47.3 vs. 23 %) than in wild-type EGFR patients, raises the question about enhanced chemosensitivity of EGFR-mutated tumors, and is object of further researches.

EURTAC (Erlotinib vs. standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive NSCLC) phase III trial [21] included NSCLC patients with EGFR mutations in metastatic first-line setting. Patients ($n = 173$) were randomized in a 1:1 ratio to receive erlotinib or standard chemotherapy (platinum plus docetaxel or gemcitabine). Median PFS was 9.7 months for patients treated with erlotinib and 5.2 months for those treated with chemotherapy (HR 0.37, 95 % CI 0.25–0.54; $p < 0.0001$). ORR with erlotinib was 64 and 18 % for chemotherapy. Median OS did not differ significantly between the two arms.

As a result of these TKIs studies, erlotinib and gefitinib were approved in a first-line setting of advanced NSCLC with EGFR somatic-sensitizing mutations.

EGFR mutations: drug sensitive and drug resistant

In lung cancer, EGFR mutations occur in exons encoding the ATP-binding pocket of the kinase domain (exon 18 to 21). The most relevant drug-sensitive mutations are deletions in exon 19 and point mutations in exon 21 (L858R). Taken together, they account for approximately 85 % of EGFR mutations. These mutations are oncogenic and intrinsically active, and they provide receptor-increased affinity from gefitinib and erlotinib over ATP [22]. Other drug-sensitive mutations are reported at much lower prevalence such as G719X (3 %), L861X (2 %), and exon 19 insertions (1 %) [23]. On the other hand, drug-resistant mutations have been defined; these resistance mutations can appear simultaneously with a drug-sensitive mutation or as an acquired event during TKIs treatment. The most noteworthy are: L747S and D761Y in exon 19, T790M and insertions in exon 20 [24], and T854A in exon 21 [25] (Fig. 2).

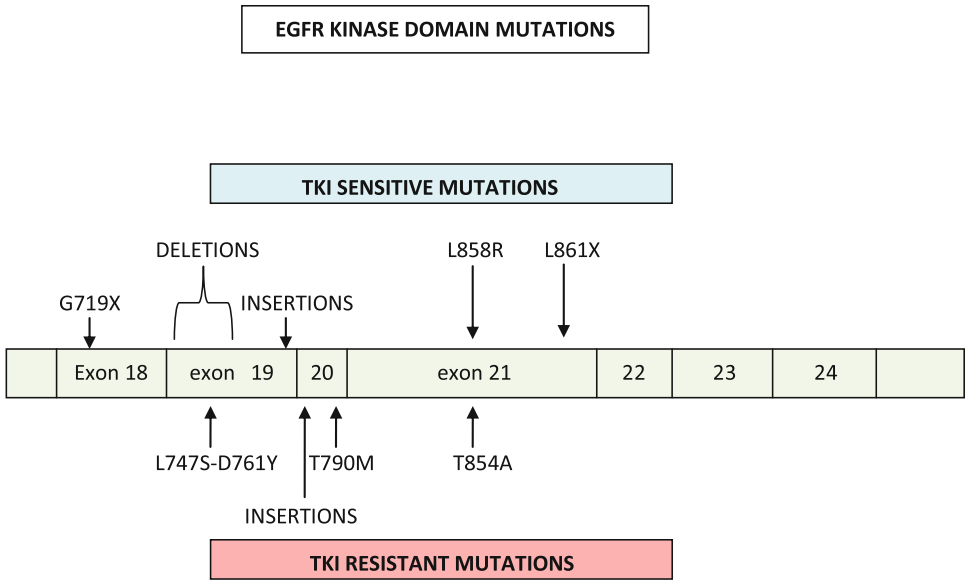

Fig. 2 EGFR kinase domain major mutations are represented; sensitizing mutations to reversible EGFR TKI (at the *top*) and resistant mutations to reversible EGFR TKI (*below*)

Prevalence of EGFR mutations varies among ethnicities: approximately 50 % of adenocarcinomas of East Asia harbor EGFR mutations; about 7–16 % of patients from an unselected population of North America and Europe have these somatic mutations [26]. EGFR mutations can be found in all histological subtypes. In a Spanish study [27], lung cancers from 2,105 patients were screened for EGFR mutations and they were found in 350 patients (16.6 %); mutations were more frequent in women (69.7 %), in patients who had never smoked (66.6 %) and in adenocarcinomas (80.9 %). EGFR mutations have also been described in squamous cell carcinoma, although its prevalence is about 3.5 % in different series [28, 29].

TKIs have demonstrated an increase in ORR and PFS compared with chemotherapy in patients with NSCLC and EGFR-sensitizing mutations in a first-line setting [14, 30]. No randomized prospective studies have yet shown that EGFR TKIs prolong OS compared with chemotherapy (mainly because once the patients in the chemotherapy arm suffer disease progression, they can benefit from switching to a TKI therapy). But there is a growing body of evidence that patients with EGFR-sensitizing mutations treated with TKIs have higher survival rates, longer than 2 years, with a median PFS among 9.2–13.1 months and an ORR ranging from 58 to 83 % [31]. Unfortunately, about 30 % of these patients do not respond to TKIs therapy and the responders will inevitably develop acquired treatment resistance [32, 33].

Conversely, a small proportion of patients whose tumors respond to TKIs have no evidence of EGFR mutations [34]. As mentioned before, NSCLC cells can release EGF ligands, establishing EGFR autocrine loops. If this loop is dependent on continued EGFR signaling and inhibited by TKIs, it could be an explanation for why some WT EGFR tumors can respond to these therapies. Taken together these considerations, we must hypothesize that more factors than EGFR mutations confer sensitivity to EGFR inhibition, such as other Erb receptors and ligands [35].

Table 1 Mechanisms of EGFR TKI resistance

Primary resistance	Acquired resistance
Kras mutations	Secondary EGFR mutation (T790M)
NF1 rasGAP mutations	
PTEN loss	c-met amplification (with–without T790M)
Concurrent secondary EGFR mutation	PI3K pathway mutations
EML4-ALK translocation[a]	Phenotypic transformation:
	– EMT
	– Transformation into other lung cancer histology
HER2 kinase domain mutation[b]	BRAF mutations
	IGF1R-mTOR-HSP90
	VEGF overexpression

[a] EML4-ALK translocation and EGFR mutation are, almost always, mutually exclusive

[b] HER2 kinase domain mutation and EGFR mutation are, almost always, mutually exclusive

EGFR mutations and primary or acquired resistance

Primary resistance

The three major mechanisms involved in primary EGFR TKIs resistance are: KRAS mutations, PTEN losses and concurrent T790M mutation (Table 1; Fig. 3). KRAS

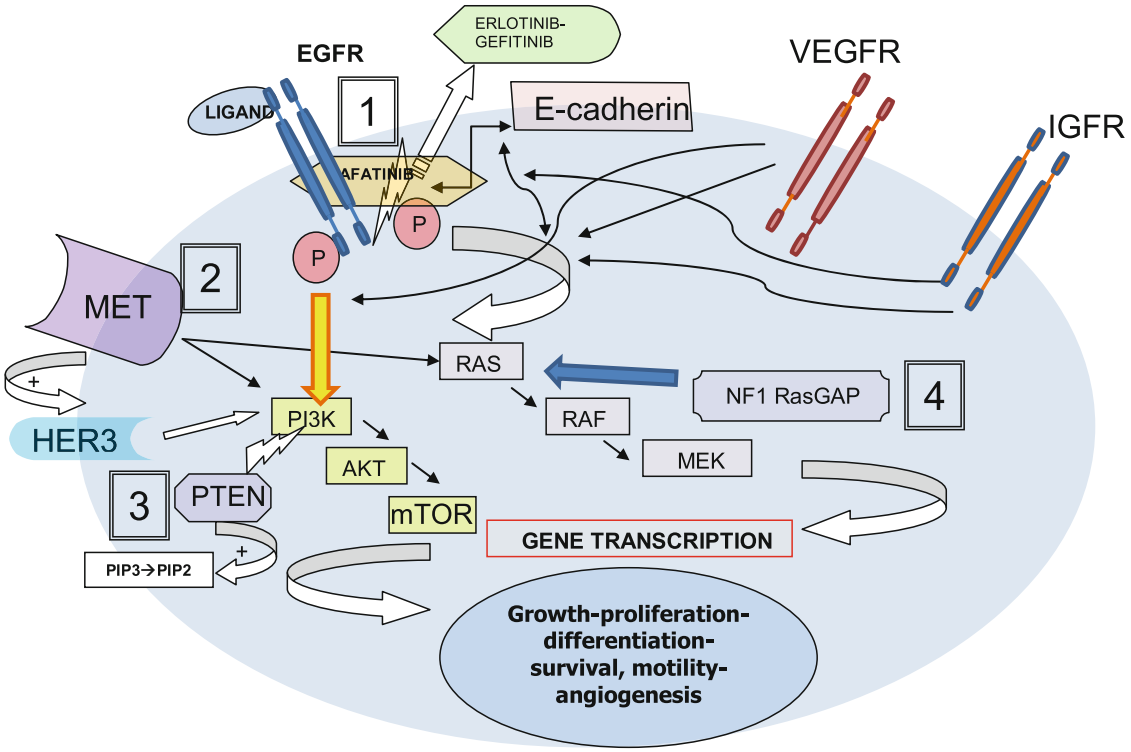

Fig. 3 EGFR primary and acquired resistance. *1* EGFR drug-resistant mutations increase tyrosine kinase affinity for ATP, which competitively displaces erlotinib–gefitinib from receptor. Afatinib covalently and irreversibly binds a cysteine residue in EGFR to the amino acid position 797, leading to EGFR kinase activity inhibition even in presence of an EGFR T790M mutation. *2* MET amplification can stimulate HER3 dependent activation of PI3K and also RAS downstream pathway. *3* PTEN downregulates PI3K signaling by dephosphorylating PIP3 into PIP2. The loss and/or mutation of PTEN lead to hyperactive PI3K pathway. Mutations in PI3K protein can also stimulate this molecular pathway. *4* RAS-RAF-MEK1 mutations can lead to persistent activation of downstream pathways. NF1 RasGAP increases KRAS persistent activation

mutations and PTEN losses are, almost always, mutually exclusive with EGFR mutations, which could confer TKI resistance in EGFR wild-type tumors.

KRAS: the Kirsten rat sarcoma viral oncogene homolog

Mutations in KRAS, NRAS, BRAF and MEK1 rarely occur in EGFR-mutant tumors. Oncogenic driver mutations of this pathway downstream of EGFR in lung cancer appear with the following frequencies: KRAS 15–30 %, NRAS 1 %, BRAF 3–5 % [36] and MEK1 1 % [37].

KRAS mutations are found in approximately 30 % of lung adenocarcinomas and 5 % of squamous carcinomas. These mutations occur primarily at codon 12 or 13 of exon 2, and they are associated with a history of tobacco use. A metaanalysis of 28 studies evaluating NSCLC found that mutant KRAS was a negative prognostic indicator for OS with a HR of 1.30 for all studies, and of 1.52 in adenocarcinomas studies [38]. KRAS was initially thought to be a poor prognostic marker of survival, but data are contradictory [39].

In addition, NF1 RasGAP disabling mutations [40] increase the occurrence of persistent RAS activation in NSCLC to approximately 40 %. Retrospective analyses suggest the association between KRAS mutations and a lack of response to EGFR TKI therapy, but it remains unclear whether there is a relationship between KRAS mutations and EGFR TKI PFS and OS [41].

In an analysis of approximately 200 lung cancer sample tumors with acquired resistance to TKIs, no RAS or MEK1 mutations were identified, but two BRAF mutations (V600E/G469A) were described. This opens a possible way towards BRAF inhibition as a strategy to overcome resistance to EGFR TKIs [42].

PTEN

Loss of function mutations in PTEN leads to a hyperactive PI3K pathway [43] which is hypothesized to cause de novo TKIs resistance. Loss of PTEN permits high level of AKT activity independent of TK receptor status, causing stimulation of downstream pathways [44]. Indeed, inhibition of AKT should lead to overcome TKIs resistance in this subset of patients. Several PI3K-AKT inhibitors are in clinical development for NSCLC [45].

Concurrent T790M mutation

EGFR mutation at T790M accounts for more than 50 % of acquired TKI resistance [46]. The coexistence of a drug-sensitive and a drug-resistant EGFR mutation has become a trending topic in Oncology. Are T790M mutations second events which appear after a long exposure to EGFR TKIs, or do they pre-exist before TKI therapy? Are both statements true? Is there a T790M cell population selection after prolonged exposure to TKI drugs which can emerge as the dominant tumor clone, conferring EGFR TKIs resistance? [47].

A retrospective study included 73 lung cancer samples before TKI treatment, and they were analyzed for correlation with TKI response [48]. In this sample, 31.5 % of patients had pretreatment T790M mutation using Matrix-Associated Laser Desorption Ionization- Time of Flight Mass Spectrometry (MALDI-TOF MS) detection method, whereas only 2.7 % of patients had T790M by direct sequencing. All T790M mutations detected by direct sequencing were also positive by MALDI-TOF MS. All T790M mutations coexisted with EGFR-sensitizing mutations; of the 56 patients with EGFR mutations, 23 had also de novo T790M and showed significantly shorter PFS compared with 33 patients without T790M mutation (median PFS, 6.7 vs. 10.2, 95 % CI 1.044–3.292, $p < 0.05$). However, the TKI RR of patients with T790M was not different from those without this mutation, in concordance with other reports.

In a retrospective subgroup analysis of EURTAC [49], 123 patients with available pretreatment tumor tissue were reanalyzed for the concomitant presence of T790M and sensitizing mutation with Taqman assay. The T790M mutation was detected in 21/64 (32.8 %) patients in the erlotinib arm and 26/59 (44.1 %) in the chemotherapy arm. PFS was 12.1 months for patients with mutant T790M in the erlotinib arm, 8.8 months for patients without T790M in the erlotinib arm, 6.3 months for patients with mutant T790M in the chemotherapy arm, and 4.5 months for patients without T790M in the chemotherapy arm ($p < 0.0001$). Excitement about maximum clinical benefit in patients with double mutation treated with erlotinib was expressed by the authors.

The variability observed between studies when comparing different mutation testing methods opens the question about the ideal test that should be done to detect mutations in NSCLC. Using high-sensitive detection methods, the T790M mutation is detected in up to 68 % of rebiopsied patients [50]. Clinical EGFR mutation test should be able to include T790M mutation not only after TKI progression.

Acquired resistance

For the last several years, many have been the potential mechanisms related to the acquisition of TKIs treatment resistance (Table 1). Most outstanding mechanisms are: secondary mutations in exons 19 and 20 of the TK domain of EGFR, MET amplification, PI3K pathway mutations and phenotypic transformation. Unknown mechanisms underlying TKIs resistance constitute about 30 % [51] (Fig. 3).

Secondary mutations

Different secondary mutations in the TK domain of the EGFR have been related to acquisition of resistance to reversible EGFR TKIs. The most relevant is T790M of exon 20, which is detected in about 50 % of NSCLC tumors harboring resistance to erlotinib–gefitinib. This point mutation at the gatekeeper position T790 of exon 20 generates a substitution of threonine with a bulkier residue, methionine, which sterically hinders drug binding. T790M also increases the EGFR kinase affinity for ATP, which competitively displaces erlotinib–gefitinib from receptor [52].

Those patients who harbor T790M acquired resistance mutations have longer survival than patients with another acquired resistance mechanism [53]. EGFR-mutated NSCLC cell lines, with or without T790M mutation, exhibited more sensitivity to irradiation in contrast to WT EGFR [54]. Data derived from retrospective studies and preclinical researches show that T790M mutation does not appear as a ubiquitous mutation and that possesses a dynamic behavior [55]. The first observation means that, many times, T790M emerges as an acquired resistance mutation in some tumor lesions whereas other lesions continue responding to TKIs treatment; the second observation refers to a curious phenomenon named oncogene addiction [56].

It has been widely known that human cancers usually evolve through a multistage process with accumulation of mutations and epigenetic changes that affect different genes. But in many occasions, only one or few of these disturbances can promote tumor cell growth and survival. The concept of oncogene addiction refers to the dependence of a cancer cell on one overactive gene or pathway for its development. The apparition of secondary mutations as T790M in EGFR TK domain in patients treated with gefitinib–erlotinib is an example of cancer growth and survival dependence on "one and only" genetic event.

This cancer growth selection under one well-defined genetic way can be interpreted as the *tumor Achilles heel*, becoming a molecular target that may be candidate to specific drug development.

Met amplification

Mesenchymal–epithelial transition factor (MET) receptor tyrosine kinase can be mutated or overexpressed in lung

cancer. The gene for MET is located on chromosome 7q and its ligand is the hepatocyte growth factor (HGF). Downstream molecules involved in the regulation of MET induce motility, migration and regulation of tumor angiogenesis [57].

MET amplification has been reported in 20 % of EGFR TKI-naïve patients, but its primary role seems to be related to acquired TKIs resistance. It has been shown that amplification of MET generates erlotinib–gefitinib resistance by stimulating HER3 dependent activation of PI3K, even in presence of activating EGFR mutations or by secondary amplification of RAS downstream pathway [58].

Concomitant presence of T790M mutation and MET amplification has been described in patients under treatment with TKIs, so concurrent inhibition of both mechanisms could be critical for overcoming resistance [59].

Mutations in PI3K protein family have been also described as an acquired resistance mechanism to EGFR TKIs in about 4 % of NSCLC [60].

Phenotypic transformation

Acquired resistance to EGFR TKIs may also appear through tumor transformation to other histological types:

- Epithelial to mesenchymal transition (EMT): mesenchymal status is related with intrinsic resistance to TKIs. EMT is a process in which epithelial cells acquire phenotypic characteristics of mesenchymal cells, such as down-regulation of E-cadherin and up-regulation of vimentin, fibronectin and n-cadherin [61]. E-cadherin, a cell surface transmembrane molecule, is involved with EGFR in activating downstream signaling pathways and its repression renders cell insensitive to TKIs therapy [62].
- Transformation into other lung cancer histological type: most recently reports [51] publish transformation of EGFR-mutant adenocarcinoma to small cell lung carcinoma (SCLC); another case report of transformation to a high-grade neuroendocrine carcinoma with combined features of SCLC and NSCLC with neuroendocrine morphology has been published [63]. Most patients with SCLC transformation after TKIs progression can respond to platinum-etoposide therapy [64].

Role of rebiopsy in resistant EGFR tumors

Emerging data support the value of tumor rebiopsy in NSCLC patients after TKI progression. A retrospective analysis [65] with NSCLC patients whose tumors became resistant to treatment with TKIs, included biopsies taken before and after TKI treatment in patients with either EGFR mutation or who had demonstrated a duration of response to TKIs of more than 24 weeks. Comparison of pre- and post-TKI biopsies showed that the frequency of T790M mutation was 47.6 % following TKI treatment; two patients lost T790M and exon 21 mutations that were recorded prior to treatment. A total of 13 patients developed EGFR exon 19 mutations. Furthermore, 17 patients following TKI treatment showed both T790M and exon 19 mutations. Finally, one patient with an exon 19 deletion in the pre-TKI treatment biopsy exhibited transformation to SCLC.

Taken together these data and described resistance mechanisms, rebiopsy can be a powerful arm and must be considered especially in patients who become resistant to TKI, since it can add information on tumor characteristics that may directly affect treatment decision and define mechanisms under the development of resistance.

Other resistance mechanisms under evaluation

EML4-ALK translocation confers resistance to EGFR TKI therapy, although this could be explained by the absence of EGFR-sensitizing mutations [66]. EGFR mutations and EML4-ALK translocation were initially thought to be mutually exclusive, but coexistence of these two alterations has been reported. In fact, concomitant EML4-ALK translocation has been detected in 15.8 % of patients of Eurtac trial [67]. Crizotinib is an ALK kinase inhibitor which also targets c-MET. A potential role of crizotinib in MET amplification mediated EGFR TKI resistance is being explored [68].

HER2/neu kinase domain mutations are found in approximately 1–4 % of lung adenocarcinomas. They appear typically in women and never smokers with no concurrent EGFR mutations. This subset of patients is also object of research with targeted therapies [69].

Overcoming resistance to TKIs

In order to define more specifically TKIs acquired resistance concept, selection criteria have been proposed [70]. All patients should have the following:

- Previously received treatment with a single-agent EGFR TKI (gefitinib–erlotinib).
- Either or both the following: a tumor that harbors an EGFR mutation known to be associated with drug sensitivity or objective clinical benefit from treatment with an EGFR TKI as defined by either documented partial or complete response (RECIST or WHO) or significant and durable ($\geq$6 months) clinical benefit (SD as defined by RECIST or WHO) after initiation of erlotinib or gefitinib.

– Systemic progression of disease (RECIST or WHO) while on continuous treatment with gefitinib or erlotinib within the last 30 days.
– No systemic therapy between cessation of gefitinib–erlotinib and initiation of new therapy.

EGFR-mutated tumors tend to grow slowly despite evidence of RECIST progression suggesting that some tumor cells remain sensitive to TKI. Patients with EGFR-mutant tumors can display a disease flare with symptomatic and radiographic progression after stopping TKIs, while improvement is noted after restarting the treatment. Moreover, clinical observations in patients with acquired clinical resistance to EGFR TKIs have shown a symptomatic disease flare attributable to disease progression after treatment discontinuation. Using TKI therapies, NSCLC tends not only to decrease in size but also to undergo morphologic changes on computed tomography (CT), such as ground glass opacity, cavitations and attenuation changes within target lesions [71]. Therefore, the response to TKIs may be inadequately assessed by RECIST criteria.

These clinical and radiographic observations call for the development of additional response criteria which complements RECIST [72]. Combination with positron emission tomography (PET) showing the tumor metabolic behavior could be useful in defining response criteria.

Second-generation irreversible EGFR TKIs

Erlotinib and gefitinib reversibly block tyrosine-kinase receptor; theoretically, an irreversible block should be more effective. Activity of reversible EGFR TKIs in T790M mutant samples is limited or even non-existent [73]. In order to get higher affinity for the tyrosine-kinase domain, second-generation irreversible EGFR TKIs are emerging.

- AFATINIB: a highly selective and irreversible ErbB family inhibitor of both EGFR and HER2 kinases. In cell assays, afatinib has a similar potency than gefitinib for inhibiting L858R EGFR and comparable to lapatinib inhibiting HER2; however, afatinib has shown 100-fold greater activity in preclinical models against L858R-T790M EGFR double mutants than gefitinib. Afatinib covalently and irreversibly binds a cysteine residue in EGFR to the amino acid position 797, leading to EGFR kinase activity inhibition even in presence of an EGFR T790M mutation [74]. Afatinib has also shown preliminary clinical activity in patients NSCLC patients harboring a HER2 mutation, present in approximately 2–4 % of adenocarcinomas [75].

– LUX-LUNG3 phase III trial [76] results have been recently reported. This is a randomized study of afatinib vs. pemetrexed–cisplatin as first-line treatment for patients with advanced lung adenocarcinoma harboring EGFR-sensitizing mutations; 345 patients were randomized to afatinib 40 mg or chemotherapy. Significant improvement in median PFS (11.1 months in afatinib group vs. 6.9 months in chemotherapy arm, HR 0.58, 95 % CI 0.43–0.78; $p = 0.0004$) was observed. In 308 patients with common mutations (del19/L858R), median PFS was 13.6 vs. 6.9 months, respectively (HR 0.47, 95 % CI 0.34–0.65, $p < 0.0001$). ORR was significantly higher with afatinib (56 vs. 23 %; $p < 0.0001$).

LUX-LUNG3 represents the largest trial in EGFR mutation-positive lung cancer patients. A potential weakness of these afatinib trials is that no data about concomitant or acquired T790M mutations have been reported; this should provide an opportunity to dispel some doubts about the initial promising afatinib activity in T790M-mutated cells.

Combined EGFR targeting with afatinib and cetuximab has induced near complete responses in T790M murine models, with no responses with erlotinib and cetuximab combination. This observation has lead into further research in afatinib and cetuximab combination in patients with acquired resistance to erlotinib or gefitinib, with a theoretical benefit of double-inhibition blockade of EGFR and HER2. In a phase II dose trial [77] with 26 patients with TKI acquired resistance, disease control was observed in all of them including 36 % of confirmed partial responses.

- DACOMITINIB: a pan-HER inhibitor that irreversibly and covalently binds to the ATP domain of each of three kinase-active member of the HER family: EGFR, HER2 and HER4. In patients with progressive NSCLC after treatment with an EGFR TKI and one or more chemotherapy regimens, dacomitinib showed antitumor activity in phase I and II trials [78]. Phase III trials are ongoing.

Met-acquired resistance

Several agents are being developed to specifically target MET, including monoclonal antibodies (onartuzumab) and TKIs (tivantinib). Dual inhibition of MET and EGFR has also demonstrated activity in preclinical models of EGFR-resistant NSCLC [79].

In a phase II trial [80] in previously treated patients with EGFR TKI naïve advanced NSCLC, 167 patients were randomized to erlotinib plus tivantinib or erlotinib plus placebo. PFS and OS significant improvement in planned subset analysis were reported in patients with non-

squamous histology who were treated with erlotinib plus tivantinib. Biomarker studies showed that among non-squamous tumors 75 % were MET-positive by IHC, compared with only 12 % of squamous tumors. Based on these data, a randomized, double-blind, placebo controlled phase III study [81] of tivantinib plus erlotinib vs. placebo plus erlotinib, in patients who have received 1–2 prior lines of chemotherapy but TKI naïve, was designed. Unfortunately, the trial has been discontinued since no OS benefit has been found after an interim analysis [82].

Onartuzumab (MetMab) is a humanized monoclonal antibody that binds to MET preventing HGF ligand binding and blocking downstream signaling. In a phase II study [83], patients with MET-positive tumors (IHC) who received erlotinib plus onartuzumab had significant reduction in the risk of death and disease progression compared with erlotinib alone. A phase III trial in MET-positive NSCLC patients previously treated with at least one but no more than two prior lines of chemotherapy, but TKI naïve, is open for accrual [84].

Other therapeutic targets in overcoming resistance

Resistance to anti-EGFR therapy has been also supposed to be secondary to increased vascular endothelial growth factor (VEGF) expression. These two signaling pathways are independent but are closely interlinked [85]. EGF and TGFα both induce VEGF expression via activation of EGFR in cell culture models. It has been postulated that overactivation of pathways driving VEGF expression independently of EGFR might result in resistance to EGFR inhibitors due to the inability of these agents to down-regulate VEGF to a non-angiogenic "point of no return", because EGFR inhibition does not substantially inhibit angiogenesis.

BELIEF, a phase II prospective trial of erlotinib and bevacizumab in patients with advanced NSCLC and sensitizing EGFR mutations with or without T790M mutations at diagnosis, represents an ongoing trial with VEGFR and EGFR inhibition approach [86].

Vandetanib, an inhibitor of VEGF receptor, EGFR and RET signaling, was compared with placebo [87] in patients with advanced NSCLC who had received no more than two prior chemotherapy regimens and had experienced treatment failure with an EGFR TKI. The trial did not demonstrate any OS benefit and serious adverse events rate was high.

The insulin-like growth factor-1 receptor (IGF-1R) is also interconnected with EGFR pathway. Preclinical models [88] with acquired EGFR TKIs treatment resistance have shown up-regulation of IGF-1. Mammalian target of rapamycin (mTOR) [89] and heat shock protein 90 (HSP90) chaperone [90] are also other targets under evaluation in this setting.

Alternation of reversible TKIs and combination of chemotherapy plus TKI

Based on the premise that erlotinib is administered at maximum tolerated dose (MTD) whereas gefitinib is approximately given at one-third of this, there is some evidence derived from small clinical series that erlotinib

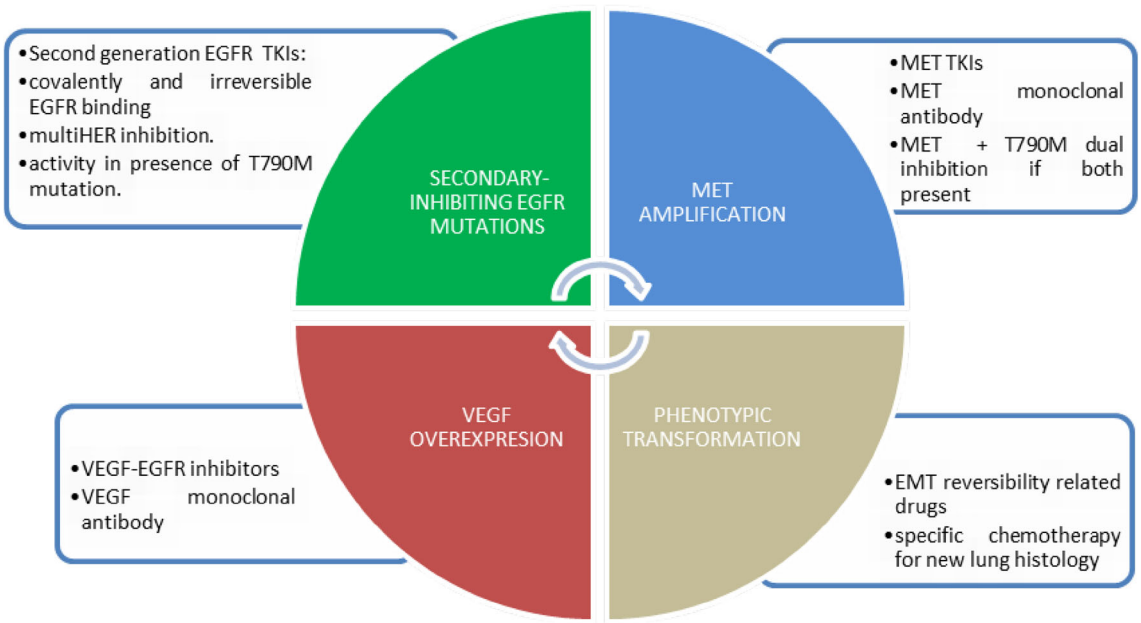

Fig. 4 Some noteworthy EGFR TKI resistance mechanisms and potential therapeutical approaches

could be used after progression on gefitinib [91–93]. Pooled analysis of the reports of erlotinib after failure of gefitinib for NSCLC showed that erlotinib might produce clinical benefits in patients who had shown long SD on prior gefitinib therapy [94].

EGFR-mutant patients treated with EGFR TKIs often present progression of one or more tumor lesions while others remain unchanged or continue responding. Taking into account that acquired resistance mutations have a dynamic behavior and having in mind the oncogene addiction model, it could be hypothesized that a TKI treatment-free interval might result in restoration of gefitinib–erlotinib sensitivity. As a consequence of the foregoing considerations, another explored approach is the association with a chemotherapeutic agent at the time of TKI resistance acquisition. A retrospective study [95] of gefitinib plus paclitaxel in patients with gefitinib progression disease provided a response rate of 13 %, a PFS of 4.2 months and OS of 8.1 months. LUX-LUNG5 is an ongoing randomized, open-label, active-controlled trial of afatinib plus weekly paclitaxel vs. investigator's choice of chemotherapy following afatinib monotherapy in NSCLC patients failing erlotinib or gefitinib [96].

Conclusions

Understanding the biological basis of lung cancer development is essential in order to design therapeutical approaches. EGFR TKIs primary and acquired resistance mechanisms are complex and heterogeneous, but definition of EGFR onco-addicted lung cancer into molecular subgroups could better clarify researches in targeted specific drugs (Fig. 4).

TKIs constitute the best choice of treatment in NSCLC patients with sensitizing EGFR mutations; once their disease is under progression a critical scenario opens: a clinical trial or chemotherapy seem the most reasonable options in daily clinical practice. Many are the open issues in the setting of TKIs resistance: which is the best treatment option? What "kind of progression disease" is the patient suffering? Which are the mechanisms under resistance acquisition? Is a rebiopsy worthy? Are RECIST/WHO criteria adequate in EGFR-mutant patients? Is PET with CT scan a better evaluation instrument than CT scan alone?

EGFR second-generation TKIs are called to play a role in NSCLC treatment options but their best place in the therapeutic arsenal is an object of discussion, since their results are similar to those of erlotinib–gefitinib. Clinical evaluation of potential drugs in patients with EGFR TKI-resistant disease is warranted.

Conflict of interest None.

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