Mechanisms of Resistance to Target Therapies in Non-small Cell Lung Cancer

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Contents

Abstract

Targeted therapies are revolutionizing the treatment of advanced non-small cell lung cancer (NSCLC). The discovery of key oncogenic events mainly in lung adenocarcinoma, like *EGFR* mutations or *ALK* rearrangements, has changed the treatment landscape while improving the prognosis of lung cancer patients. Inevitably, virtually all patients initially treated with targeted therapies develop resistance because of the emergence of an insensitive cellular population, selected by pharmacologic pressure. Diverse mechanisms of resistance, in

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particular to EGFR, ALK and ROS1 tyrosine-kinase inhibitors (TKIs), have now been discovered and may be classified in three different groups: (1) alterations in the target (such as *EGFR* T790M and *ALK* or *ROS1* mutations); (2) activation of alternative pathways (i.e. *MET* amplification, *KRAS* mutations); (3) phenotype transformation (to small cell lung cancer, epithelial–mesenchymal transition). These basic mechanisms are informing the development of novel therapeutic strategies to overcome resistance in the clinic. Novel-generation molecules include osimertinib, for EGFR-T790M-positive patients, and new ALK-TKIs. Nevertheless, the possible concomitant presence of multiple resistance mechanisms, as well as their heterogeneity among cells and disease localizations, makes research in this field particularly arduous. In this chapter, available evidence and perspectives concerning precise mechanisms of escape to pharmacological inhibition in *oncogene-addicted* NSCLC are reported for single targets, including but not limited to EGFR and ALK.

Keywords
ALK • EGFR • NSCLC • Resistance mechanisms • ROS-1 • T790M

1 Introduction

Lung cancers currently figure among the most frequent tumor diagnoses and are the most relevant in terms of mortality worldwide (Siegel et al. 2016).

Before the year 2000 the dichotomy between small cell and non-small cell lung cancer (SCLC and NSCLC, respectively) was sufficient to address treatment strategies. Further histologic definition within NSCLC (squamous cell carcinoma and adenocarcinoma) was therefore recognized as clinically meaningful (Scagliotti et al. 2008). Since the last decades, molecular sub-typing of NSCLC (with an almost exclusive regard to adenocarcinoma) is providing a drastic refinement in the detection of alterations suitable of specific inhibition, generating a dramatic evolution in patients' management. Such aberrations (whose incidence in western population is showed in Fig. 1), in general mutually exclusive, normally represent the very funding oncogenic event (Gainor et al. 2013). The targeting of such altered tyrosine-kinase (TK) receptors by means of specific inhibitors (TKIs, actively competing against ATP-binding) usually generates extremely rapid and profound tumor responses, defining thus far the scenario of *oncogene addiction* (Lynch et al. 2004; Paez et al. 2004; Kwak et al. 2010). In this field, the superiority of targeted agents over standard chemotherapy, in the advanced setting, is at this point evident (Mok et al. 2009; Solomon et al. 2014).

Albeit targeted therapies are revolutionizing the treatment of advanced NSCLC, sooner or later resistance appears in virtually every patient. Molecular treatment exhaustion denotes the emergence of a cellular population insensitive and selected by the pharmacologic pressure. In parallel to the crucial recognition of specific mechanisms on the diagnostic samples, the detection of molecular reasons explaining treatment resistance at the moment of disease progression, obtaining

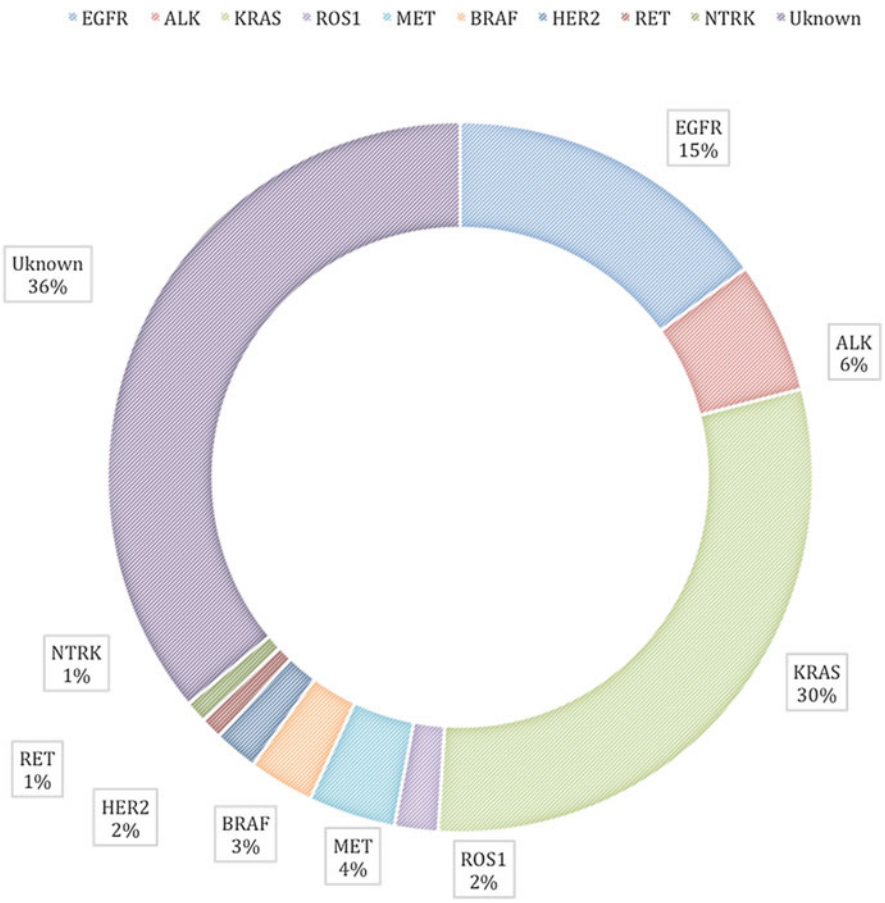

Fig. 1 Distribution of molecular aberrations responsible for *oncogene addiction* in lung adenocarcinoma affecting Western populations

of novel tumor material (*re-biopsy*) harbours a similar pivotal importance. The role of re-biopsies in the clinical setting is currently gaining more and more relevance due to the development of novel-generations Epidermal Growth Factor Receptor (EGFR)-TKIs, like osimertinib (AZD9291), active against T790M *EGFR* mutation, whose emergence is the most common mechanism of resistance to first-and second-generation anti-EGFR compounds (Kobayashi et al. 2005; Cross et al. 2014; Mok et al. 2016) (see next paragraphs).

Patterns of resistance to tailored therapies are shared among different activating aberration, and lessons regarding targets rare in lung cancers can be driven from other tumors. The general molecular ways lung cancer cells find to escape directed targeting are resumed in Table 1. The possible concomitant presence of multiple mechanisms, as well as their heterogeneity among cells and disease localizations (Suda et al. 2016; Hata et al. 2015), makes research in this field particularly

Table 1 Schematic description of the main resistance mechanisms to targeted treatments in non-small cell lung cancer

Mechanisms	Examples	Oncogene
Alterations in the target	*EGFR* T790M mutations	*EGFR*
	ALK TKD mutations – *ALK* amplification	*ALK*
Activation of bypass pathways	*MET* or *HER2* amplification	*EGFR*
	EGFR hyperactivation	*ALK*
Morpho-phenotypic evolutions	EMT and transformation from ADC to SCLC	Both

TKD tyrosine kinase domain, *EMT* epithelial–mesenchymal transition, *ADC* adenocarcinoma, *SCLC* small cell lung cancer

arduous. Available evidence and perspectives concerning precise mechanisms of escape to pharmacological inhibition in *oncogene-addicted* NSCLC are reported for single targets in the next paragraphs.

2 Resistance Mechanisms to EGFR-Driven NSCLC

Mutations in the *EGFR* gene are the most frequent oncogenic drivers in NSCLC, reported in approximately 10–15% of Caucasian NSCLC patients (Rosell et al. 2009) and 30–50% of Asians ones (Mok et al. 2009). The development of EGFR-TKIs, such as erlotinib, gefitinib (belonging to the first generation) and afatinib (second generation), shaped a great shift in the therapeutic management of *EGFR*-mutated NSCLC patients resulting in improved response rate (RR), progression free survival (PFS) and quality of life compared to first-line platinum-based chemotherapy (Mok et al. 2009; Rosell et al. 2012; Yang et al. 2015).

Unfortunately, prognosis remains unfavourable because of the occurrence of treatment resistance.

However, the identification of some mechanisms of resistance improved the therapeutic chances of these patients. In particular, the point mutation p.Thr790Met (T790M) occurring in *EGFR* exon 20 is responsible of resistance in about 50–60% of the patients when progression occurs (Sequist et al. 2011). Recently, the third-generation TKI osimertinib improved outcomes in patients harbouring this new mutation (Mok et al. 2016). Some other molecular resistance mechanisms have already been identified, but other information are needed to better understand and effectively overcome resistance to EGFR-TKIs in the remaining 40–50% lacking T790M mutation. Although exciting survival data and response rates have been registered in patients treated with osimertinib, acquired resistance unfortunately still occurs also during this therapy (Minari et al. 2016). Here, we will review principle mechanisms of resistance described during NSCLC treatment with both first-/second- and third-generation EGFR-TKIs.

2.1 Resistance to First- and Second-Generation EGFR-TKIs

Today erlotinib and gefitinib, together with the second-generation afatinib, are recognized as the standard first-line therapy in NSCLC patients with activating *EGFR* mutations (Mok et al. 2009; Rosell et al. 2012; Yang et al. 2015). Despite these important results, some patients with confirmed mutations in the EGFR-TK domain do not respond to EGFR-TKIs at all (de novo/*intrinsic resistance*). The remaining *EGFR*-mutated patients, after favourable and prolonged responses, inevitably exhibit disease progression (*acquired resistance*), usually after 10–14 months of treatment. Although the large majority of evidence concerns tumor evasion of targeted treatments represented by erlotinib and gefitinib, afatinib exhaustion seems to share the same molecular mechanisms (Campo et al. 2016). Several mechanisms of resistance have been identified and they may be classified in three different groups, as indicated in the introduction: (1) *EGFR* mutations; (2) activation of alternative pathways; (3) phenotype transformation (Table 1 and Fig. 2).

2.1.1 Preclinical Evidence and Clinical Relevance of Resistance Mechanisms

Mechanisms of primary resistance are still not fully understood, but several cases of de novo inefficacy of EGFR-TKIs are the consequence of the presence of

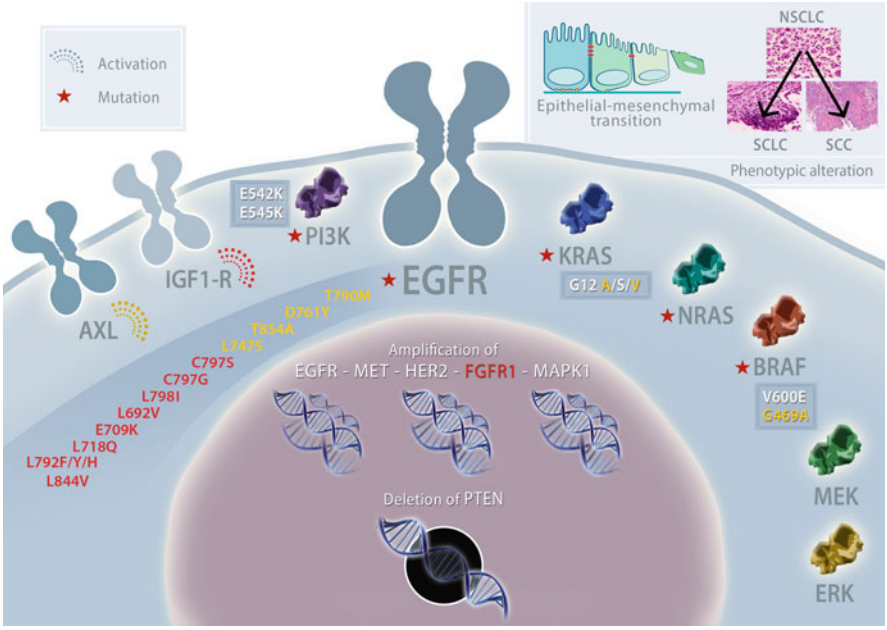

Fig. 2 Mechanisms of resistance to first/second (*yellow*) and third (*red*)-generation EGFR-TKIs; shared mechanisms among the three TKI generations are depicted in *white*. *NSCLC* non-small cell lung cancer, *SCLC* small cell lung cancer, *SCC* squamous cell carcinoma

non-sensitive *EGFR* mutations. Exon 20 insertions, which represent the 1–10% of the total number of *EGFR* mutations, adding residues at the N-lobe of EGFR (M766 to C775) in particular in the C-helix (A767 to C775), frequently reduce affinity for EGFR-TKIs (Yasuda et al. 2013). New sequencing technologies are able to detect cases of concomitant (double or multiple) *EGFR* mutations. Patients with a combination of typical and atypical mutations reported less favourable outcomes compared to patients with a single typical mutation (Wu et al. 2011). Also the coexistence of different driver alterations in other genes, such as *ALK* rearrangements and *KRAS* mutations, resulted associated with worse prognosis after EGFR-TKI treatment in *EGFR*-mutated NSCLC (Ulivi et al. 2016).

The most common mechanism of resistance is the development of acquired T790M *EGFR* gene mutation (Sequist et al. 2011), a secondary point mutation in exon 20, engendering the substitution of methionine (T) for threonine (M) at codon position 790, that sterically prevents the EGFR-TKI binding in the TK domain (TKD), allowing the ATP-mediated activation of the receptor (Kobayashi et al. 2005). Nevertheless, T790M mutation has been also identified as a de novo mutation (Inukai et al. 2006). In this case of primary resistance, it is predictive for poor survival outcomes under EGFR-TKI treatment (Su et al. 2012). Moreover, the T790M impact on responsiveness to EGFR-TKI therapy may depend on the proportion of pre-treatment EGFR T790M-positive clones (Hata et al. 2016).

Third-generation EGFR-mutant selective inhibitors (such as osimertinib and rociletinib) have been developed for patients whose cancers acquire the T790M mutation. These third-generation EGFR-TKIs realize selective inhibition of activating as well as T790M alterations, by means of an irreversible covalent binding to the target while sparing wild-type EGFR (Cross et al. 2014), with important efficacy results and reduced toxic effects (Mok et al. 2016; Jänne et al. 2015; Sequist et al. 2015).

Other rare resistance *EGFR* point mutations including D761Y, T854A and L747S have been reported in less than 10% of mutated NSCLC patients. The mechanism underlying resistance conferred by these mutations is still unclear (Nguyen et al. 2009).

The activation of alternative pathways is now recognized as a different mechanism of resistance (Niederst and Engelman, 2013; Yu et al. 2013a).

The *MET* gene amplification is the second most common mechanisms of acquired resistance, affecting about 5–20% of NSCLC patients during EGFR-TKI treatment, irrespective of the T790M mutation status (Sequist et al. 2011; Engelman et al. 2007). *MET* amplification, accompanied by HGF (MET ligand) autocrine signalling, drives resistance to EGFR-TKIs acting upon molecular elements regulating critical intracellular pathways (Engelman et al. 2007; Turke et al. 2010). MET inhibition has proven to be effective in cell lines with *MET* gene amplification and many preclinical and clinical data demonstrate that contemporary inhibition of MET and EGFR may be a strategy to overcoming resistance (Engelman et al. 2007; Bahcall et al. 2016; Gainor et al. 2016a).

HER2 amplification is a rare event in lung adenocarcinoma at diagnosis, accounting for about 1–2% of cases, but it has been reported in up to 13% of

NSCLC with acquired resistance to EGFR-TKIs (Yu et al. 2013a; Takezawa et al. 2012), whereas resulting absent in other series (Sequist et al. 2011). Mutated EGFR has the tendency to heterodimerize with HER2, the resulting heterodimers being resistant to degradation (Takezawa et al. 2012). Therefore, HER2 heterodimerization could support EGFR-TKIs resistance in presence of both T790M mutation and *HER2* amplification itself as acquired mechanisms of drug exhaustion.

Boosting of cell signalling pathways due to activation of BRAF, PIK3CA and AXL has been proposed as mechanism of drug resistance in cancer cells and in *EGFR*-mutated NSCLC patients, in which their emergence can be overall detected in up to 20–25% of cases (Sequist et al. 2011; Ohashi et al. 2012; Wang et al. 2014; Zhang et al. 2012).

A third resistance mechanism is the phenotypic transformation of lung cancer cells. Histological transformation in SCLC has been observed after the development of acquired resistance to EGFR-TKI in about 3–14% of patients (Sequist et al. 2011; Yu et al. 2013a). The mechanism underlying this histological modification is still not completely known: minor pre-existent cells under the selection pressure of EGFR-TKIs could originate SCLC cells or adenocarcinoma cells could transdifferentiate in SCLC cells (Oser et al. 2015); alternatively, SCLC cells could develop from multi-potent pre-existing stem cells (Oser et al. 2015). Whatever the funding cellular evolution, the loss of Rb protein seems a common and necessary event for this kind of transformation (Niederst et al. 2015a).

Loss of E-cadherin expression and upregulation of mesenchymal proteins such as vimentin, fibronectin and N-cadherin are the main features of epithelial–mesenchymal transition (EMT). In the EMT setting, AXL upregulation and alterations in the Hedgehog pathway have been recently recognized as mechanisms of resistance to targeted agents in EGFR-mutated NSCLC (Zhang et al. 2012; Thomson et al. 2005).

Moreover, transformation from adenocarcinomas to squamous cell carcinomas during the administration of anti-EGFR molecules has been reported as a mechanism of acquired drug resistance (Haratani et al. 2016).

Anyway, the cause of resistance remains still unknown in 18–30% of NSCLC patients resistant to anti-EGFR targeted therapy (Sequist et al. 2011; Yu et al. 2013a).

2.1.2 Detection of T790M Mutation

According to current guidelines, after progression to first-line EGFR-TKI treatment, carrying out a new biopsy to identify the molecular mechanism of acquired resistance and to select patients for targeted therapies is a reasonable procedure (Novello et al. 2016). The feasibility and utility of re-biopsies have been evaluated in several clinical experiences (Mok et al. 2016; Campo et al. 2016; Arcila et al. 2011).

However, serial tumor sampling to monitor cancer evolution is not always feasible in clinical practice. An alternative approach in NSCLC patients may be indeed the use of the so-called *liquid biopsy*, whereby circulating cell-free tumor DNA (ctDNA), DNA fragments passively released into the blood by primary cancer cells, or circulating tumor cells (CTCs), viable or apoptotic cells released from the

primary tumor, can be analysed in the peripheral blood to detect *EGFR* mutations (Crowley et al. 2013; Douillard et al. 2014). Dynamic changes of *EGFR* mutational status in ctDNA seem to predict the clinical outcome to EGFR-TKI treatment (Tseng et al. 2015). A meta-analysis showed a sensitivity of 61% and a specificity of 90% for blood (plasma and serum) analysis compared to tissue evaluation in identifying *EGFR* mutations with a concordance rate of 79% (Mao et al. 2015).

Many studies confirmed the utility and validity of plasma DNA in detection T790M mutation in patients with NSCLC who progressed under EGFR-TKI therapy (Mok et al. 2016; Sundaresan et al. 2016; Remon et al. 2017). According to results of many studies, this method it is today approved by the FDA (http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/DeviceApprovalsandClearances/Recently-ApprovedDevices/ucm519922). Considering the level of sensitivity, when a liquid biopsy is negative for the detection of *EGFR* T790M, this result should be confirmed on tissue biopsy specimen (Oxnard et al. 2016). In addition, a study recently demonstrated satisfying agreements in T790M status definition between urine, plasma and tissue (Reckamp et al. 2016); further urine-based tests are indeed under study.

As mentioned before, T790M mutations account for up to 60% of resistant cases to first- and second-generation EGFR-TKIs (Sequist et al. 2011; Yu et al. 2013a). The remaining T90M-negative cases, for which, in addition, molecular treatment strategies are less developed, can be less characterized by liquid biopsy, with special regard to morpho-phenotypic changes. Recently nevertheless, a high quote of *KRAS* activating mutations has been uncovered in ctDNA in EGFR-mutant NSCLC patients progressing to first or second-generation TKIs (Del Re et al. 2016).

2.1.3 Potential Strategies to Overcome Resistance

Some clinical strategies have indeed been developed in order to *deal with* or overcome resistance to first- and second-EGFR-TKIs.

Because of cancer heterogeneity, once the onset of resistance is manifest, some clones may continue to remain sensitive to EGFR-TKIs, whose continuation can slow down disease progression. For these reasons, in selected patients with slow-growing and low-volume disease, progression in non-critical or asymptomatic sites, no clinical deterioration or intolerable toxicity, first-line EGFR-TKI treatment can be continued beyond progression, as several retrospective studies and some prospective experience showed (Park et al. 2016; Yap et al. 2017).

In the case of clinical progression in circumscribed localization, disease behaviour reflects the spatial heterogeneity of resistance. Retention of the targeted treatment with the addition of local approaches (including surgical resection or radiotherapy) to the dimensionally increasing lesions results a suitable option in order to achieve long-term disease control, acting directly against the resistant counterparts while maintaining active EGFR suppression (Weickhardt et al. 2012; Yu et al. 2013b).

Brain metastases interest around 20% of *EGFR*-mutated NSCLC patients at diagnosis, while 30–60% of them, during an effective EGFR-TKI administration,

develop central nervous system lesions, often representing the isolated site of disease recurrence (Heon et al. 2010; Khalifa et al. 2016). If the progression after first-line EGFR-TKI therapy is characterized by the development of isolated brain metastases, stereotactic radiotherapy or surgery, when possible, is recommended, while multiple lesions require whole-brain radiotherapy. In order to keep extracerebral disease control, EGFR-TKI treatment should be continued (Khalifa et al. 2016).

These reported are effective clinical ways to delay the requirement of novel (cytotoxic or targeted) treatment. In virtually every patient indeed, disease progression not allowing such approaches sooner or later occurs. The research of T790M mutation on tumor specimens or ctDNA is crucial, both for the quote of patients harbouring it and for the actual possibility to overcome it with the novel molecules of third generation, such as osimertinib, rociletinib, HM61713 (olmutinib), ASP8273, EGF816 and PF-06747775. The clinical development of rociletinib and olmutinib has been recently interrupted and ASP8273, EGF816 and PF-06747775 are under early investigation.

Osimertinib is an oral, irreversible EGFR-TKI that is selective for both activating and T790M-resistance mutations (Cross et al. 2014), with significant activity against central nervous system metastases too (Mok et al. 2016; Ballard et al. 2016). In the phase 1 trial (AURA) the RR for osimertinib in patients with T790M-positive tumors was 61%, with a median PFS of 9.6 months (Jänne et al. 2015). These findings were confirmed in a pooled analysis of two subsequent phase 2 studies (Yang et al. 2016), one of which recently published (AURA2) (Goss et al. 2016). On the basis of these results, FDA approved osimertinib in T790M-positive NSCLC patients. A confirmatory, randomized, open-label, international phase 3 trial (AURA3) was conducted and osimertinib showed significantly greater efficacy than platinum plus pemetrexed chemotherapy in patients with T790M-positive cancers after progression under first- or second-generation EGFR-TKIs (Mok et al. 2016). Median PFS for osimertinib was 10.1 months, compared to 4.4 months for chemotherapy (HR: 0.30; 95% CI 0.23–0.41; $p < 0.001$) (Mok et al. 2016). Under the AURA development, osimertinib became the standard of care in second-line treatment for *EGFR*-mutated patients harbouring the T790M mutation.

At the time of progression for patients who are not candidate to osimertinib due to the absence of *EGFR* T790M resistance mutation, different therapeutic options have been or are under investigation. The randomized phase III IMPRESS trial compared gefitinib with versus chemotherapy alone in 265 EGFR-mutated NSCLC resistant to first-line gefitinib (Soria et al. 2015). The underlying objective was to sound out the potential contribution of maintaining inhibition of the driver molecule in addition to standard cytotoxic treatment. No benefit in survival was observed when gefitinib was associated with chemotherapy, suggesting that the EGFR-TKI should be discontinued in resistant patients when the switch to chemotherapy is required (Soria et al. 2015, 2016).

In order to overcome the resistance mediated by specific bypass mechanisms, targeting the detected drivers of resistance itself in combination with EGFR-TKIs may be a sound therapeutic possibility. In particular, the use of MET inhibitors in

combination with EGFR-TKI recently revealed as a promising strategy for *EGFR*-mutated and *MET* amplified NSCLC. Cabozantinib, capmatinib and tepotinib reported significant results in terms of disease response if associated with anti-EGFR agents in this subgroup of NSCLC (Bahcall et al. 2016; Wu et al. 2016; Soo et al. 2015).

In cases of rapid systemic progression, performing a new biopsy is recommended because in presence of a phenotypic transformation to SCLC, to squamous cell carcinoma or when EMT is evident, the use of the chemotherapy could be more beneficial than the use of target therapies.

2.2 Resistance to Third-Generation EGFR-TKIs

The introduction of third-generation EGFR-TKIs resulted in a further outcome improvement for a selected group of NSCLC patients. Nevertheless, despite the high RR and the significant prolongation of survival, after 9–13 months, unfortunately, acquired resistance occurs again (Mok et al. 2016; Jänne et al. 2015; Goss et al. 2016). Several (and not fully recognized) underlying molecular mechanisms have been described (Minari et al. 2016).

2.2.1 Preclinical Evidence and Clinical Relevance of Resistance Mechanisms

In the case of third-generation EGFR-TKIs too, we can classify the mechanisms of resistance in three different categories: (1) EGFR-dependent mechanisms; (2) activation of alternative pathways; (3) phenotypic transformation (Table 1 and Fig. 2).

The emergence of tertiary *EGFR* mutations has been repeatedly reported in the presence of acquired resistance to third-generation TKIs and it has been well characterized in cell lines models (Minari et al. 2016). The *EGFR* p.Cys797Ser (C797S) mutation in the exon 20 is the most common mutation responsible for resistance to osimertinib. Firstly, C797S was identified in ctDNA of 6 out of 15 (40%) patients progressing to osimertinib in the AURA phase I/II study (Thress et al. 2015). It seems also responsible of acquired resistance to other third-generation EGFR-TKIs such as HM61713 and WZ4002, but it is rare after rociletinib (Niederst et al. 2015b; Ercan et al. 2015; Chabon et al. 2016). The substitution of a cysteine with a serine in the position 797 of the tyrosine kinase domain reduces the inhibitory effect of third-generation TKIs by interfering with their covalent binding to EGFR (Thress et al. 2015). Interestingly, according to preclinical evidence, the location of C797S mutation among other *EGFR* alleles (in *cis* vs. in *trans*) may affect the efficacy of subsequent treatments (Niederst et al. 2015b).

After the report of this first mutation responsible of resistance third-generation EGFR-TKIs, several other single-site alterations, such as L718Q and L844V, have been reported in patients and in cellular models treated with osimertinib or other third-generation TKIs (Minari et al. 2016). Importantly, liquid biopsy confirmed its value in detecting such mutations in ctDNA, reinforcing its importance as a clinical

tool (Thress et al. 2015; Ercan et al. 2015; Chabon et al. 2016; Piotrowska et al. 2015).

Again, EGFR-independent mechanisms of resistance during third-generation TKI treatment can emerge. *HER2* amplification was discovered in a NSCLC patient with disease progression after 12 months of osimertinib in the AURA trial (Planchard et al. 2015). It appeared to be mutually exclusive with *EGFR* T790M mutation, as described for first-generation TKIs (Takezawa et al. 2012), and not associated with C797S. Similar findings were reported in other patients treated with osimertinib (Minari et al. 2016), while in the case of resistance to rociletinib, *HER2* amplification was associated with T790M persistence (Chabon et al. 2016). *MET* amplification was first reported in a single case of NSCLC after 10 months of osimertinib treatment, in the absence of T790M or C797S mutations (Planchard et al. 2015), and it was documented too as a mechanism of acquired resistance, both in preclinical in vitro models and clinical cases (Ortiz-Cuaran et al. 2016; Ou et al. 2016a).

In preclinical studies of acquired resistance to osimertinib, an increased dependence on RAS signalling was reported. *NRAS* mutations, including a novel E63K mutation, and *NRAS* or *KRAS* amplification have been described as mechanisms of acquired resistance to osimertinib (Eberlein et al. 2015). The emergence of three *KRAS* activating mutations (p.G12A, p.Q61H and p.A146T), alone or in combination with other resistance mechanisms, has been reported after rociletinib (Chabon et al. 2016). Moreover, at the time of progression, p.E542K and p.E545K mutations in *PIK3CA* gene have been described in five patients treated with rociletinib (Chabon et al. 2016). Other reported resistance mechanisms include *BRAF* p.V600E mutation (Oxnard et al. 2015) and *EGFR* amplification (Chabon et al. 2016; Piotrowska et al. 2015).

Finally, after third-generation TKIs too, in some cases resistant tumors showed phenotypic changes, such as SCLC transformation or EMT (Piotrowska et al. 2015; Kim et al. 2015; Ham et al. 2016).

2.2.2 Potential Strategies to Overcome Resistance

Currently, different therapeutic strategies to overcome the above-described heterogeneous resistance mechanisms to third-generation TKIs are under development.

A new era of fourth-generation TKIs is coming (Minari et al. 2016). EAI045 is the first of a new class of inhibitors, able to overcome T790M and C797S mutations, being selective against mutant-EGFR while sparing the wild-type forms. The combination of EAI045 and cetuximab showed efficacy in mouse models of lung cancer carrying EGFR L858R/T790M/C797S mutations (Jia et al. 2016).

Combinations with third-generation TKIs are being investigated in several studies to avoid the occurrence or overcome resistance (Minari et al. 2016). The association of the MEK inhibitor trametinib with the third-generation EGFR-TKI WZ4002 was able to prevent the emergence of resistance in *EGFR*-mutant lung cancer models (Tricker et al. 2015). The association of osimertinib and another MEK inhibitor, selumetinib, prevented the onset of resistance in cellular lines and

reported in vivo cancer regression in an *EGFR*-mutated, T790M-positive, osimertinib-resistant transgenic model (Eberlein et al. 2015).

Some evidences suggest that patients with C797S and T790M mutations *in trans* could be sensitive again to the association of first/second-generation TKIs with third-generation ones, while the *in cis* disposition results in resistance to all molecules, both alone and in combination (Niederst et al. 2015b). Moreover, the occurrence of C797S in T790M wild-type cells is responsible of resistance to third-generation TKIs, despite the sensitivity to first-generation TKIs (Niederst et al. 2015b). Patients progressing on rociletinib achieved response with osimertinib (Sequist et al. 2016), suggesting a slight different activity of the molecules developed against the T790M mutation.

For patients whose tumors undergo SCLC transformation or EMT, switching platinum-based chemotherapy could be recommended.

Surely, other escape mechanisms are likely to emerge, highlighting the importance of molecular characterization at the time of progression, aiming at the definition of the most correct therapeutic strategy.

3 Resistance Mechanisms to ALK- and ROS1-Driven NSCLC

ALK and *ROS1* rearrangements are present in approximately 4–7% and 1–2% of NSCLC, respectively (Barlesi et al. 2016; Bergethon et al. 2012). These two oncogenes share profound similarities in phylogenesis, biology, genomic sequences, profiles of pharmacological inhibition and tumor clinical features (Ou et al. 2012). Importantly, tumors driven by either ALK or ROS1 manifest similar mechanisms of resistance to targeted agents, which will be approached in parallel. Several mechanisms of drug escape have been identified and they may be classified, similarly to EGFR-TKIs, in three different groups, as indicated in the introduction: (1) involving the target (ALK or ROS1); (2) activation of alternative pathways; (3) phenotype transformation (Table 1 and Fig. 3).

3.1 Mechanisms of Crizotinib Resistance

3.1.1 Mechanisms of Crizotinib Resistance Involving ALK and ROS1

Crizotinib, firstly developed as a MET inhibitor (Kwak et al. 2010), is currently registered by FDA and EMA for patients suffering from *ALK*- and *ROS1*-rearranged NSCLC. Similarly to EGFR-driven tumors, mutations in the target have been reported as the first mechanism of resistance to crizotinib for both oncogenes (Choi et al. 2010; Awad et al. 2013). The number of reported *ALK* mutations responsible of acquired resistance is high, whereas altogether their detection is present in around 30% of clinical samples (Gainor et al. 2016b).

Concomitantly with the first report of crizotinib clinical activity (Kwak et al. 2010), Choi and colleagues described *ALK* C1156Y and L1196M mutations as responsible of acquired resistance to the drug (Choi et al. 2010). L1196 corresponds

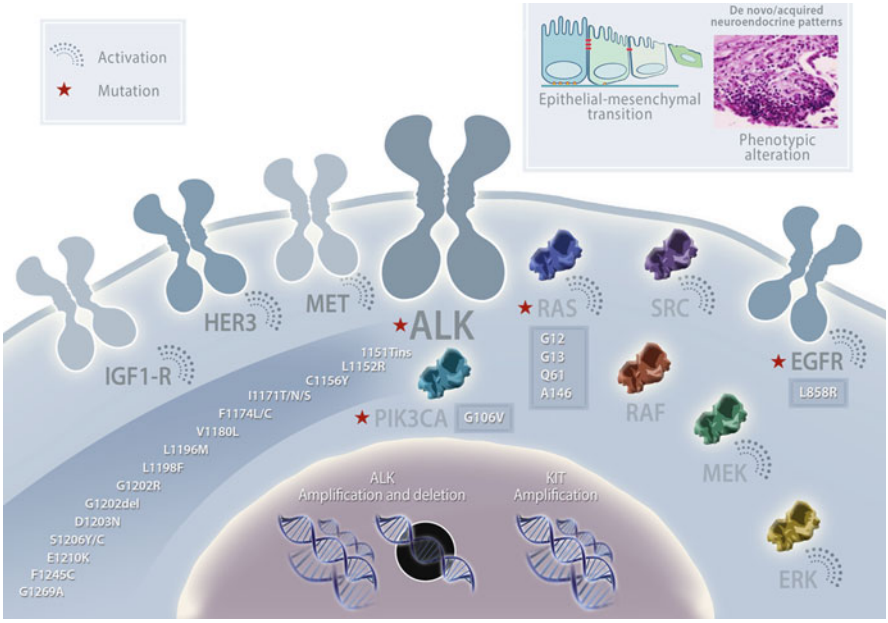

Fig. 3 Thorough representation of mechanisms of resistance to crizotinib and novel-generation inhibitors in ALK-rearranged non-small cell lung cancer. See Gainor et al. Cancer Discovery 2016 (Gainor et al. 2016b) to distinguish the differential mechanisms for single molecules

to the gatekeeper residue in ALK tyrosine kinase domain and this substitution is among the most frequently reported single-nucleotide alteration responsible of crizotinib resistance in NSCLC, together with G1269A (Gainor et al. 2016b). Biopsies obtained in *ALK-positive* NSCLC patients at the moment of disease progression to crizotinib (Katayama et al. 2012; Doebele et al. 2012) allowed the detection a conspicuous number of mutations in ALK TKD. These latter engender crizotinib resistance by means of two main mechanisms: by increasing enzymatic activity at a level not suitable of crizotinib inhibition or interfering with its binding (Gainor et al. 2016b; Friboulet et al. 2014). Considering also isolated case reports, single-nucleotide substitutions reported thus far include 1151Tins, L1152R, C1156Y, I1171N/S/T, F1174C/V, L1196M, G1202R, D1203N, S1206Y, F1245C and G1269A (Gainor et al. 2016b; Facchinetti et al. 2016a) (Fig. 3).

Due to the more limited rate of patients harbouring the oncogenic aberration and to the particularly recent introduction of crizotinib for ROS1-positive advanced NSCLC, information concerning resistance emerging in this setting is barely less abundant. Given the homology between ALK and ROS1 TKD (which share >80% sequence identity within their ATP-binding sites), every mutation reported so far to negatively affect crizotinib activity in ROS1-positive patients find its corresponding with comparison to *ALK* (Facchinetti et al. 2016b). G2032R (Awad et al. 2013), D2033N (Drilon et al. 2016a) and S1986Y/F (Facchinetti et al. 2016b), here reported in order of discovery, can be indeed aligned with the

corresponding *ALK* G1202R, D1203N and C1156Y, respectively. Moreover, the gatekeeper L2026M substitution, together with L1951R, has been recently reported in a patient biopsy after crizotinib resistance (McCoach et al. 2016). The clinical relevance of other mutations (L2155S, K2003I, L1951R and M2128V) reported in vitro only has yet to be established (Katayama et al. 2015; Song et al. 2015).

ALK activation in NSCLC is a consequence of gene fusions and the most frequently partner gene is represented by *EML4*. Several fusion variants are possible, showing slight differential sensitivity to crizotinib in cellular models (Heuckmann et al. 2012) and potentially accounting to the variable durations of disease control in the clinics (Yoshida et al. 2016; Woo et al. 2016).

Crizotinib exhaustion can depend from the amplification/copy number gain of the *ALK* gene itself (Doebele et al. 2012; Shaw et al. 2014) and the possibility of loss of the driver alteration under selective pressure has been proposed in the clinics (Doebele et al. 2012).

These mechanisms have not yet been reported in ROS1-postive NSCLC, neither in preclinical studies nor in the clinics.

3.1.2 Activation of Bypass Pathways Explaining Crizotinib Resistance

Occurrence of either *ALK* or *ROS1* rearrangements together with *KRAS* mutations in NSCLC can explain primary (Mengoli et al. 2016; Schmid et al. 2016) or acquired (Doebele et al. 2012) resistance to crizotinib. Moreover, KRAS and NRAS activation through mutations or amplifications leads to the exhaustion of first-generation inhibitors activity in ROS1-positive cellular models (Cargnelutti et al. 2015).

EGFR signalling has been reported as the responsible of crizotinib resistance in a non-negligible rate of patients-derived biopsies and in cellular models (Katayama et al. 2012; Doebele et al. 2012). Similarly to what observed for RAS alterations, EGFR signalling could augment, with or without the evidence of classical activating mutations (Katayama et al. 2012; Doebele et al. 2012; Kim et al. 2013). Several experiences indeed shown the amplification of *EGFR* gene and implementation of EGFR phosphorylation have been functionally related to acquired resistance to crizotinib, in both ALK- and ROS1-dependent NSCLC (Katayama et al. 2012; Song et al. 2015; Kim et al. 2013; Davies et al. 2013).

KIT gene amplification or activating mutations in *ALK* and *ROS1*-rearranged NSCLC patients can, respectively, explain the acquisition of crizotinib resistance (Katayama et al. 2012; Dziadziuszko et al. 2016).

Moreover, IGF1R, SRC and MEK/ERK activation can mediate resistance to specific inhibitors in ALK-dependent cell models, suggesting thus far the potential of combinatorial strategies in the clinics (Lovly et al. 2014; Crystal et al. 2014; Hrustanovic et al. 2015).

3.1.3 Further Mechanisms of Crizotinib Resistance

The largest part of ALK-positive NSCLC patients exposed to crizotinib experiences intracranial disease progression in spite of extra-cerebral disease control. Pharmacokinetics issues concerning the low blood–brain barrier penetration of the

compound account for its reduced, albeit present, activity in CNS lesions (Costa et al. 2011). Data concerning ROS1-driven disease are still too limited to infer such similar conclusions.

Morpho-phenotypic tumor changes in ALK-positive disease are more limited than *EGFR* mutated, while in *ROS1*-rearranged cells they are limited to preclinical reports (Song et al. 2015). The neuroendocrine phenotypes of NSCLC can be responsible of either de novo crizotinib resistance (Omachi et al. 2014) or for the exhaustion of drug efficacy after initial responses, as transformation from adenocarcinoma to SCLC (Caumont et al. 2016).

In vitro data suggests EMT contribution to the establishing of resistance to ALK inhibitors (Kogita et al. 2014). Experimental data are sustained by clinical reports (Kobayashi et al. 2013).

3.1.4 Overcoming Crizotinib Resistance

In the very recent years, several other molecules have been developed aiming to maintain specific inhibition of ALK and ROS1 when crizotinib runs out of activity. Second-generation compounds include ceritinib, alectinib and brigatinib, while lorlatinib belongs to the third-generation of drugs. If ceritinib, brigatinib and lorlatinib are characterized by half maximal inhibitory concentration (IC50) values suitable of clinical application in both ALK- and ROS1-driven diseases, according to cellular assays alectinib exclusively hits ALK (Gainor et al. 2016b; Facchinetti et al. 2016b; Davare et al. 2015).

The mentioned inhibitors shown crucial properties, such as the ability to inhibit the target more potently than crizotinib, the activity against multiple mutant forms of ALK and ROS1 and the good brain penetration, confirming their relevant activity in the setting of crizotinib resistance (Facchinetti et al. 2016a, 2017). Data from clinical trials and patients' cohorts suggests the clear advantage of administering new-generation compounds after crizotinib (Facchinetti et al. 2016a, 2017). There is moreover an efficacy gradient from the less recent to the newest inhibitors, as the latest are more potent and active against a larger number of mutations in the targets conferring resistance, as long as the brain penetration and the selectivity increase (Gainor et al. 2016b; Zou et al. 2015).

Among the cited novel inhibitors, only ceritinib and lorlatinib ostensibly harbour a significant role as ROS1 TKIs, considering the reduced potency of brigatinib against the wild-type and mutated forms of the enzyme (Chong et al. 2017), together with the inefficacity of alectinib.

Other wide-spectrum tyrosine-kinase inhibitors (e.g. cabozantinib, foretinib, entrectinib) shown activity against ROS1 and/or ALK (Facchinetti et al. 2017). The current availability of the mentioned specific inhibitors with important anti-ALK activity makes questionable the utilization of the latter ones in ALK-driven diseases. Among these less-specific molecules, cabozantinib only could have a role, as the sole drug potentially active against the G2032R *ROS1* mutation, the most frequent mechanism of crizotinib resistance reported so far, albeit in very small series (Katayama et al. 2015; Gainor et al. 2016c). Nevertheless, the toxicity spectrum of cabozantinib at the systemic concentrations required to inhibit mutant

ROS1 forms, still leave doubts about its potential clinical proposition; beside, all other crizotinib-resistance mutations seem to be overcome by the more manageable drug lorlatinib (Facchinetti et al. 2017).

3.2 Resistance to Next-Generation ALK and ROS1 Inhibitors

Recently, Gainor and colleagues reported the results from a wide series of ALK-positive tumor biopsies obtained at progression to crizotinib or second-generation inhibitors (Gainor et al. 2016b). Some of the codons involved in TKD mutations were previously unreported in the clinics, as E1210K, conferring crizotinib resistance, and V1180L, occurring after alectinib administration and already approached in in vitro studies (Gainor et al. 2016b; Katayama et al. 2014).

After ceritinib and alectinib, amino-acidic substitutions mediating resistance were observed in more than 50% of the samples, compared with the 20–30% target alterations responsible of crizotinib exhaustion (Gainor et al. 2016b). Although post-brigatinib biopsies were limited in the series, an enrichment in *ALK* G1202R mutation was reported after the onset of resistance for all the three new inhibitors compared to post-crizotinib samples. Other *ALK* mutations responsible of new-generation inhibitors resistance, unreported after crizotinib, are emerging, as reported in a patient developing ceritinib exhaustion due to the G1123S substitution (Toyokawa et al. 2015), underlying the differential selective pressure exerted by the inhibitors.

PIK3CA G106V activating mutation was detected in an alectinib-resistant specimen, thus allowing to envisaging the involvement of the AKT-mTOR pathway in resistance (Redaelli et al. 2016), as seen in *EGFR*-mutant NSCLC (Sequist et al. 2011).

Bypass signalling activation mediated by IGF1R, HER3 (with the concomitant overexpression of its ligand neuregulin-1) and MET has been proven as mechanism of resistance to alectinib in cellular models (Isozaki et al. 2016).

Morpho-phenotypic changes driving EMT were clearly depicted in one ceritinib-resistant sample and present, with different levels of intensity, in up to 42% (five out of 12) specimens, often in the presence of ALK mutations (Gainor et al. 2016b). Two cases of SCLC transformation were reported in alectinib-resistant tumors (Fujita et al. 2016; Takegawa et al. 2016).

Great interest was raised by the first and, to date, the only report of specific lorlatinib resistance in an ALK-rearranged NSCLC patient. Tumor cells, already harbouring the crizotinib and ceritinib resistant mutation C1156Y, developed the previously unknown L1198F substitution, determining in vitro resistance to all available ALK inhibitors except for crizotinib (Shaw et al. 2015). Administration of the first-generation compound led indeed to disease response. Data concerning resistance to new-generations inhibitors in ROS1-positive NSCLC models or patients are still lacking.

4 Resistance Mechanisms to Targeted Drugs in NSCLC Driven by Other Oncogenes

As several other targets and corresponding pharmacological compounds are emerging in lung cancer, mechanisms of resistance in this new field are rising and lessons can be inferred from tumor models other than NSCLC, sharing driving molecular aberrations and specific inhibition.

4.1 MET

Beside its role in mediating resistance to EGFR inhibition (Engelman et al. 2007), MET is known as a meaningful driver oncogene in NSCLC since around a decade. Nevertheless, its precise mechanisms of activation, harbouring biological and clinical relevance, have been profitably elucidated in the last 2 years (Drilon et al. 2017). *MET* gene amplification needs a precise definition for achieving a meaningful role in predicting response to specific inhibitors, the most relevant one to date again represented by crizotinib (Drilon et al. 2017). Moreover, MET can be biologically activated by a newly recognized mechanism represented by the loss of its exon 14 (*exon 14 skipping*), coding for the juxtamembrane domain, that leads to a meaningful increase in MET signalling by means of the decrease of its degradation (Awad 2016). As in this case MET TKD remains intact, crizotinib is indeed active (Drilon et al. 2017). Nevertheless, mutations occurring in *MET* TKD (D1228N, D1228V, Y1230C, the two latter founded in ctDNA too) have been recently reported as putative responsible of resistance to MET inhibitors in the clinics (Bahcall et al. 2016; Heist et al. 2016; Ou et al. 2016b). Strategies to overcome resistance to type I MET inhibitors (which preferentially bind to the active conformation of the protein, e.g. crizotinib and savolitinib) with type II compounds (which preferentially bind to the inactive molecule conformation, e.g. cabozantinib and capmatinib) yield an in vitro and clinical crucial meaning (Bahcall et al. 2016). If MET activation can explain resistance to EGFR inhibitors, as seen above, the reverse situation has been reported, with the onset of crizotinib resistance in a MET-driven NSCLC associated to the appearance to the activating *EGFR* L861A mutation (Benderra et al. 2016).

4.2 BRAF

BRAF-mutated NSCLC is similar in biology (in terms of role of oncogenic agent), clinical and therapeutic approaches to melanomas harbouring *BRAF* activating mutations (Nguyen-Ngoc et al. 2015). According to *BRAF*-mutant melanomas, the co-inhibition of BRAF and MEK in NSCLC generates better outcomes compared to BRAF blockade alone (Planchard et al. 2016a, b). With regard to *BRAF*-mutated lung adenocarcinoma, the onset of mutations in *KRAS*, *TP53* and *CDKN2A*

has been proposed as a resistance mechanism to the BRAF inhibitor dabrafenib in the clinics (Rudin et al. 2013).

Clear evidence concerning resistance to BRAF and MEK inhibitors in NSCLC is yet to be provided, but mechanisms could be the same observed in melanoma. Nevertheless, if *BRAF* mutations in melanoma occur for the largest part in codon V600 (of which V600E is the archetypal), in NSCLC the involvement of different BRAF activating sites in up to 50% of the cases (Nguyen-Ngoc et al. 2015). Non-V600 *BRAF* mutants are globally less potently inhibited by available anti-BRAF molecules (Gatalica et al. 2015; Noeparast et al. 2016), making the association with MEK inhibitors even more recommended.

4.3 RET

RET rearrangements drive oncogenesis in 1–2% of lung adenocarcinomas (Kohno et al. 2012). Responses to cabozantinib and vandetanib have currently been systematically recognized in phase II clinical trials (Drilon et al. 2016b; Yoh et al. 2017). Moreover, clinical activity of sunitinib (Wu et al. 2015) and alectinib (Lin et al. 2016) has been documented in RET-driven NSCLC. No clinical demonstration of molecular mechanisms of targeted treatment exhaustion is available thus far. An extensive preclinical study recently identified *RET* mutations conferring differential resistance to cabozantinib and vandetanib, while overcome by ponatinib, the most potent RET inhibitor (Huang et al. 2016), whose activity in patients is currently under study. Another experience revealed the hyperactivation of *SRC*, a central gene in focal adhesion, as a suitable mechanism of acquired resistance to dovitinib (Kang et al. 2015); specific inhibition of SRC with sarcatinib allowed the re-sensitization to RET inhibition, as robustly seen in *ALK*-rearranged models (Crystal et al. 2014).

5 Conclusions

The obtaining of the most prolonged disease control with targeted therapies represents nowadays the primary goal in oncogene-driven advanced NSCLC. The profound knowledge of the molecular mechanisms driving resistance to specific inhibitors is basilar in order to develop further treatment strategies. Nevertheless, in experiences when re-biopsies are performed once treatment exhaustion manifests, mechanistic reasons for this clinical behaviour remain biologically uncovered in up to 30–50% of the cases.

Adaptive strategies with novel inhibitors are showing outstanding results both after and in comparison with first-generation molecules when administered upfront, suggesting a scenario in which the most potent drugs would be given immediately. Nevertheless, combinatorial strategies aiming at bypass collapse, achievable with the fruitful blocking of both the primary molecular alteration and the alternative signalling tracks responsible of resistance, are still lacking. Given the relevant

potency of novel molecules against their respective targets, together with the emergence of novel resistance mutations thus far uncommon (*EGFR* C797S, *ALK* L1198F), activation of bypass pathways are expected to arise in a significant quote of cases and this therapeutic gap would need to be filled.

Inner tumor heterogeneity manifests in this field with the various mechanisms adopted by tumors to find escapes under specific therapeutic pressure, in different individuals as well as in the diverse lesions of the same patient. This represents one of the greatest issues to deal with, hampering the potential of pharmacologic developments. Strategies to face the limits imposed by biologic tumor variability are lately emerging (Suda et al. 2017).

The detection of mutations responsible of resistance to old and novel EGFR inhibitors in the blood represents a major improvement (hopefully applicable to other drivers) as a proof of principle and for practical reasons. Nevertheless, its application still requires additional adjustments.

Taken together, the evidence contained in this chapter depicts a scenario in continuous evolution, in which the search for the best-targeted treatment option in lung cancer strictly relies upon the digging towards the deeper and widest knowledge concerning biologic resistance. Questions to be solved are still copious and complex; nevertheless, the recent advances in both clinical and preclinical research, allowed by the impressive developments in experimental methods and techniques, generate enthusiasm and hope for the very next future.

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