Cancer Treatment Reviews 85 (2020) 101994

Contents lists available at ScienceDirect

Cancer Treatment Reviews

journal homepage: www.elsevier.com/locate/ctrv

The significance of epidermal growth factor receptor uncommon mutations in non-small cell lung cancer: A systematic review and critical appraisal

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ARTICLE INFO

Keywords:
EGFR
Uncommon mutations
TKIs
NSCLC
NGS
Systematic review

ABSTRACT

Uncommon epidermal growth factor receptor (*EGFR*) mutations collectively account for 10% of *EGFR* mutations, harboring heterogeneous molecular alterations within exons 18–21 with clinically variable responses to EGFR tyrosine kinase inhibitors (TKIs) in advanced Non-Small Cell Lung Cancer (NSCLC) patients. In addition, with the introduction of different NGS gene approach an improvement of *EGFR* mutations detection was reported. Today, no specific studies have prospectively evaluated uncommon sensitizing mutations in detail and no firm standard of care has been established in the first-line setting. The aim of this comprehensive review is to critically consider the clinical role of uncommon *EGFR* mutations highlighting the results of several *in vitro* and *in vivo* studies, which singly evaluated the sensitivity of uncommon mutations to currently European of Medicines Agency (EMA)-approved *EGFR* TKIs in cell lines, xenograft models and humans, in order to obtain a practical guide for refining the clinical decision-making process.

Introduction

Despite the increase of therapeutic options available in clinical setting, non-small cell lung cancer (NSCLC) still remains the leading cause of cancer-related death in both sexes worldwide, representing over 85% of all lung cancers [1]. The clinical knowledge of the epidermal growth factor receptor (*EGFR*) molecular status, emerging over a decade ago, has led to a dramatic shift in the treatment paradigm of metastatic NSCLC. The prevalence of *EGFR* mutations ranges from 10% to 15% in Caucasian patients [2] and up to 50% in East-Asian patients [3], mainly but not only with adenocarcinoma histology, female gender and quite peculiar of no smoker or former smoker patients [4]. To date, a personalized therapeutic approach based on the detection of activating mutations in the kinase domain of *EGFR* correlated directly with sensitivity to EGFR tyrosine kinase inhibitors (TKIs) in advanced NSCLC patients, represents the standard of care in the diagnostic setting of NSCLC patients [5]. So far either first- (erlotinib, gefitinib) or second-(afatinib, dacomitinib) or third-generation (osimertinib) EGFR TKIs have received the Food and Drug Administration (FDA) approval as standard first-line treatment options in *EGFR* mutated patients, leading to improved response and progression-free survival rates in several phase 3 trials [6–10]. Most *EGFR* mutations are strong predictors of response to TKIs with the most "common" type of activating or sensitizing EGFR mutation being the in-frame deletion of exon 19 around the LRE motif (amino acid residues 747–750; ~45% of *EGFR* mutations), followed by p.L858R point mutation of exon 21 (~40% of *EGFR* mutations). The remaining 10% of *EGFR* mutations appeared to harbor heterogeneous molecular alterations within exons 18–21 (so-called "uncommon" mutations) with clinically variable responses to targeted drugs and shorter survival rates when compared to classical mutations [11], even if most Phase III trials excluded patients with these mutations from the analysis to define the clinical outcome (LUX-Lung 2, 3 and 6 being notable exceptions). Although, the introduction of Next Generation Sequencing (NGS) in the clinical setting has broadened the

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https://doi.org/10.1016/j.ctrv.2020.101994
Received 11 January 2020; Received in revised form 18 February 2020; Accepted 19 February 2020

spectrum of detected mutations and medical oncologists may generally agree with the clinical usage of EGFR TKIs in this rare setting, no prospective large trials have prospectively evaluated uncommon sensitizing mutations in detail and no firm standard of care has been established. Specifically, retrospective studies of erlotinib and gefitinib seemed to show inconsistent responses [12] while a combined post-hoc analysis of phase II-III trials revealed the clinical activity of afatinib in certain type of uncommon *EGFR* mutations [13]. Even if in the FLAURA study upfront osimertinib has recently provided a statistically and clinically significant meaningful improvement in overall survival (OS) when compared only to first-generation TKIs (gefitinib and erlotinib) in *EGFR*-positive patients with classical mutations [14], small case series of osimertinib has led to equivocal benefit in NSCLC patients harboring different uncommon mutations [15]. Furthermore, very little is known about potential differences in TKI sensitivity among different *EGFR* exon19 deletions, even if recent evidences have proved equal sensitivity to first-line EGFR-TKIs [16].

Therefore, this systematic review aims to critically evaluate the clinical role of uncommon *EGFR* mutations in TKI actionability highlighting the results of several *in vitro* and *in vivo* studies, which singly evaluated the sensitivity of uncommon mutations to currently European of Medicines Agency (EMA)-approved *EGFR* TKIs in cell lines, xenograft models and humans. In addition, current evidences towards a better selection of patients who are more likely to benefit from one TKI to another are discussed in order to obtain a practical guide for refining the clinical decision-making process.

Methods

We searched for different study types including NSCLC cell lines, xenograft models or in human trials, retrospective or prospective studies, case reports, preclinical researches, and systematic reviews (Fig. 1 – PRISMA plot) regarding uncommon EGFR mutation treated with a first-, second- or third-generation TKI.

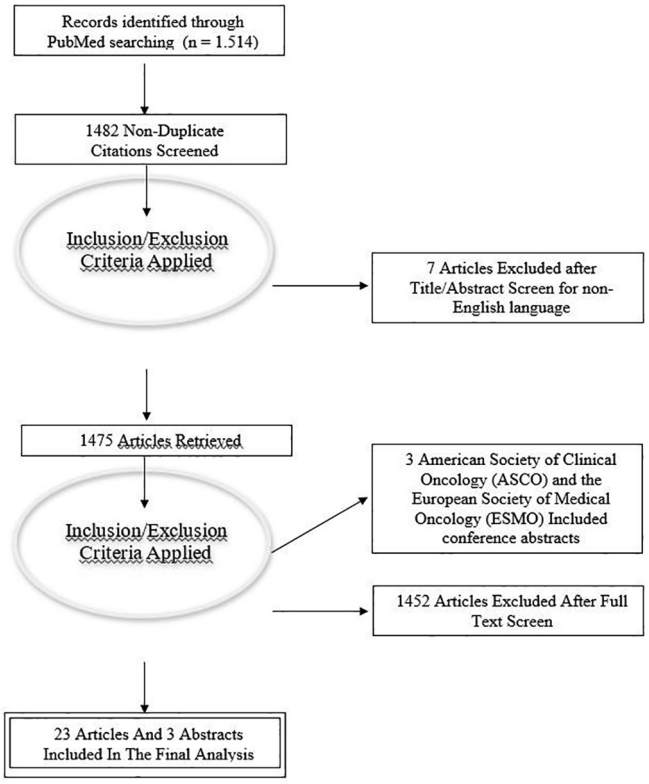

Fig. 1. PRISMA flow diagram showing the selection algorithm of retrieved papers according to the inclusion/exclusion criteria.

A systematic review was conducted up to December 2019 through Medline (Pubmed) and Cochrane-Library, without language restrictions. Some unpublished studies were searched online and results were obtained from the American Society of Clinical Oncology (ASCO) and the European Society of Medical Oncology (ESMO) conference abstracts, as well as unpublished data or results from ongoing studies available on the National Institute of Health (NIH) website (www.clinicaltrials.gov) were also considered as a source of grey literature. We used a free term strategy, producing the following search string: ("[NSCLC]" AND "[EGFR]" AND "[EGFR TKI]" AND "[EGFR GENE VARIANTS]" AND "[UNCOMMON]" OR "[RARE MUTATIONS]" OR "[MINOR MUTATIONS]") and references from relevant articles was carried out. Working in duplicate, two different reviewers (V.G. and A.G.) independently reviewed the articles by title and abstract and the full text of potentially significant articles. A third reviewer (U.M.) resolved disagreements between the other reviewers.

About inclusion criteria, data relative to *EGFR* mutations in lung cancers were extracted from the Catalogue of Somatic Mutations in Cancer (COSMIC) database, release version 90 (last access 10-8-2019). When considering *in vitro* and xenograft models studies, the half maximal inhibitory concentration (IC50), which represents the concentration of TKI that is required for 50% inhibition of *EGFR* viable cells *in vitro* and is comparable to the half maximal effective concentration *in vivo*, was evaluated to discuss the different drug sensitivity patterns among the distinct TKIs [17]. IC50 values were given and eventually converted in terms of μmol. Mutations were considered to be resistant if the tested TKI showed IC50 values > 0.1 μmol whereas considered to be sensitive with IC50 values ≤0.1 μmol [18]. As regards in human studies, progression-free survival (PFS), measured from the first day of TKI treatment until the first objective or clinical sign of disease progression or death, was matched to IC50 values and evaluated to compare the efficacy of specific TKIs among the different subsets of EGFR mutations.

Results

Exon 18

Since *EGFR* mutations in exon 18 are not fully characterized and have been reported only by a limited number of studies [19,20], it is therefore difficult to draw conclusions as to their true prevalence, molecular spectrum, clinical-pathological features and drug efficacy. Exon 18 mutations collectively account for 3–4% of all *EGFR* mutations [21], more commonly including point mutations that encompass a glycine change to serine, alanine or cysteine (p.G719S/A/C in the 97% of cases) within the codon 719, and less frequently involving the codon 709 [22]. Although displaying variable responses to TKIs and being associated with poor prognosis when compared to classic activating mutations [23], patients with p.G719A/C/S point mutations do not appear to be resistant to EGFR TKIs but do exhibit different sensitivity profile. The p.G719S mutation appeared to be less sensitive to gefitinib than erlotinib in preclinical studies [24], but response and PFS rates with first-generation TKIs did not significantly differ in the clinical setting [25]. Even if not yet evaluated in clinical studies, a consistently augmented sensitivity of p.G719S to afatinib was rather demonstrated *in vitro* and xenograft studies while osimertinib showed only intermediate sensitivity [26–28]. Likewise, p.G719C appeared to be sensitive to both first-generation TKIs from preclinical data [29,30] though with improved progression-free and overall survival rates in favor of erlotinib [31], and confirmed to be very sensitive to afatinib with IC50 values in the low micromolar range and, only in xenograft models studies, to osimertinib [27]. In contrast, p.G719A proved to be uniformly resistant to erlotinib and gefitinib in preclinical and clinical studies while showing marked sensitivity *in vitro* and in xenograft studies to afatinib and, less pronouncedly, to osimertinib [26,27]. Conversely, even if more often presenting as complex mutations with

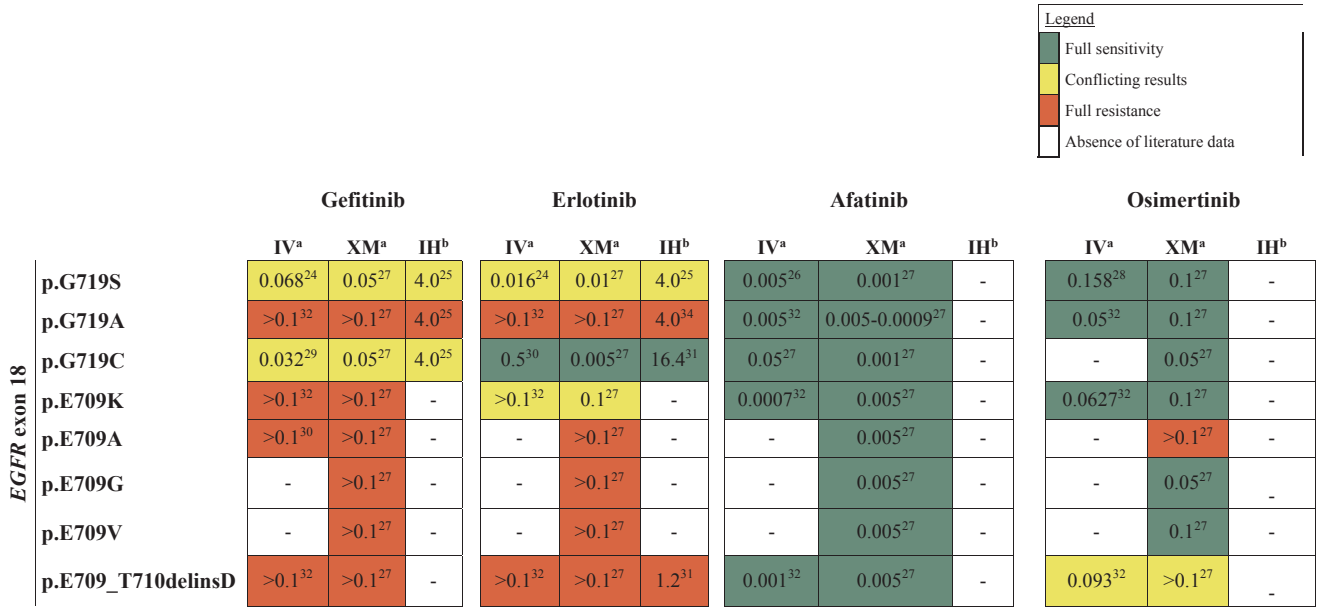

Fig. 2. Overview and assessment of EGFR TKIs activity in cell lines (IV), xenograft models (XM) and patients (IH) harboring exon 18 mutations [[a]expressed in term of micromolar (μM) concentration, [b]expressed in term of median months of progression-free survival (PFS)]. The drug sensitivity was color-coded according to the scheme indicated at the top right and was categorized as sensitive, resistant, controversial or not available based on literature data.

sensitizing p.G719S/A/C or p.L858R mutations, the vast majority of point mutations involving codon 709 (p.E709K/A/G/V) were revealed to be homogeneously resistant to first-generation TKIs [30,32] while demonstrating intense sensitivity to last-generation TKIs taken into account, mostly to afatinib. Intriguingly, p.E709K proved to be more sensitive to afatinib with at least two times lower IC50 values when compared to osimertinib, whereas p.E709A turned out to be very sensitive to afatinib in the low micromolar range and quite insensitive to osimertinib in xenograft studies [27], confirming the suggested differential activity of TKIs toward specific *EGFR* activating and resistance mutations.

Regarding exon 18 deletions, p.E709_T710delinsD has been considered to be the most common, even though it can be missed when using diagnostic commercial kits [32]. According to *in vitro* and xenograft models studies, this deletion appeared to show higher sensitivity to afatinib, even when compared to osimertinib [27,32]. However, clinical data with afatinib are scant [21] and only modest objective response rates to first-generation TKIs have been observed in selected case reports (Fig. 2) [33].

Exon 19

As previously discussed, the most frequently identified *EGFR* mutation category are exon 19 in-frame deletions, so-termed “common” mutations which account for approximately 45% of all *EGFR* alterations and are collectively known to be highly sensitive to all generations of *EGFR*-targeted TKIs. Actually, this subgroup of mutations do represent at least 30 different deletions with or without amino acid insertion/substitution, eventually displaying different sensitivity profile in both the preclinical and clinical setting [34].

The most frequently observed *EGFR* exon 19 deletion, which is nested around the conserved LRE string leading to elimination of 5 amino acids between residues 746–750 (delE746-A750) [35], showed improved objective response and PFS rates to first-generation EGFR TKIs when compared to uncommon exon 19 deletions occurring at different amino acid positions, so-called non-LRE deletions [36]. However, commercially available kits for EGFR testing used in daily practice detect only specific deletions in exon 19 and may miss uncommon deletions. Among these, an interesting finding from Kohsaka et al. study was that both afatinib and osimertinib reduced the viability of cells expressing uncommon exon 19 deletions in xenograft assays with a remarkably augmented sensitivity compared to gefitinib and erlotinib [27]. Nevertheless, clinical data directly comparing the efficacy of TKIs among minor deletions of exon 19 are lacking.

Clinical retrospective data showed that deletions occurred throughout almost the entire exon 19 amino acid string from E746 to D761 involving 16 amino acids with over half of the subtypes being complex with an accompanying insertion [37]. Indeed, a number of other exon 19 deletion mutations have been less frequently observed between amino acids 745 and 753, and associated with sensitivity to TKIs [38]. Namely, these mutations seemed to exhibit structural similarities with common exon 19 deletions and often consist of complex insertion-deletions (indels) leading to replacement of the deleted amino acids with a non-native residue (such as the p.L747-A750delinsP where a proline residue results to be introduced in substitution) [38]. Although being associated with *in vitro* and *in vivo* sensitivity to first-generation *EGFR* TKIs and afatinib with lower clinical responses comparing to classical mutation [39], several differences in sensitivity and response to EGFR TKIs among patients with these complex mutations have been reported [40]. Accordingly, p.L747_A750delinsP have shown poorer PFS rates when compared to the classical sensitizing p.E746_A750, and even to the less frequently detected p.L747_P753delinsS which actually demonstrated to be broadly sensitive to all generation TKIs in the preclinical setting [41]. No firm conclusions can be drawn since patients harboring complex deletions starting with leucine in position 747 plus insertions had significantly shorter PFS to first-generation TKIs [42] while showing no significant differences in PFS and OS to gefitinib in other retrospective series [43]. To date, preclinical findings has recently showed that the p.L747-A750 > P complex mutation is completely inhibited by low doses of afatinib whereas being less sensitive to erlotinib or osimertinib, underlining important differences among specific exon 19 complex deletions that have been also confirmed in the clinical setting [44]. Moreover, even if certain indel mutations showed a slightly increased sensitivity to first-generation TKIs in some *in vitro* studies [32], xenograft studies suggested that these genotypes were particularly responsive to afatinib and osimertinib [27], even if few clinical data appeared to favor the only use of afatinib [45] while retrospective

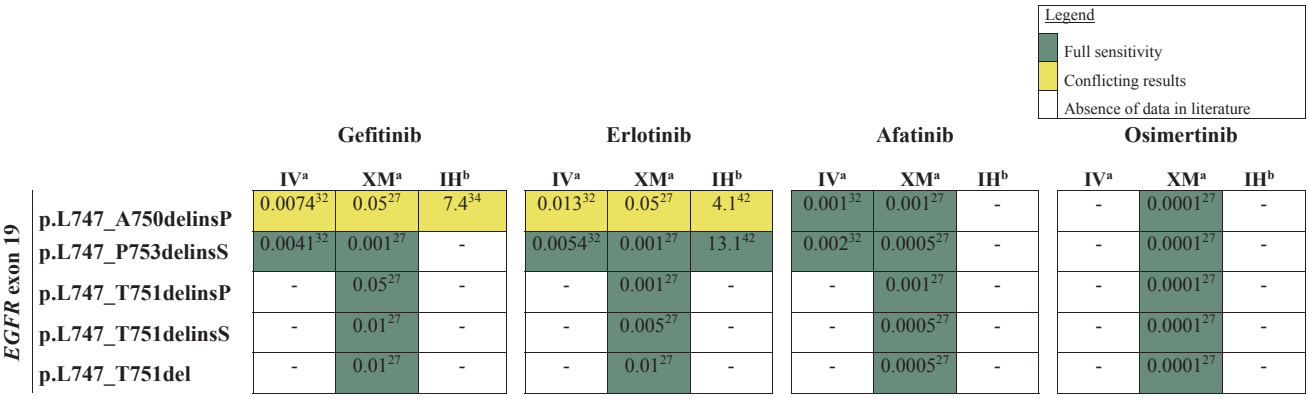

Fig. 3. Overview and assessment of EGFR TKIs activity in cell lines (IV), xenograft models (XM) and patients (IH) harboring exon 19 mutations [[a]expressed in term of micromolar (μM) concentration, b expressed in term of median months of progression-free survival (PFS)]. The drug sensitivity was color-coded according to the scheme indicated at the top right and was categorized as sensitive, controversial or not available based on literature data.

series have recently proved equal sensitivity to first-line *EGFR* TKIs among certain exon 19 deletion subtypes with further comparable activity of gefitinib and afatinib in this subset of patients (Fig. 3) [16].

Exon 20

Besides classical sensitizing mutations, exon 20 insertions represent the third most frequently detected *EGFR* mutation accounting for approximately 1.5–2.5% of all NSCLCs and 6% of all *EGFR*-mutated patients [32,46]. Among uncommon mutations, exon 20 insertions are the most prevalent and heterogeneous group of aberrations. They consist of in-frame insertions or duplications across a span of ~15 amino acids that encompass residues from 761 to 775 with the vast majority being resistant to EGFR TKIs [47] and experiencing longer PFS with platinum-based chemotherapy in retrospective studies [48]. To date those exon 20 insertions, traditionally considered to be the most prevalent and to confer resistance to all generation EGFR TKIs [49], include: p.A763_Y764insFQEA, p.A767_V769dupASV, p.V769_D770insASV and p.D770_N771insSVD.

Preclinical evidences suggested that insertions between codons 769 to 775 could lead to drug resistance whereas more proximal codons might have an activation mechanism and structure that more closely resemble those of classical sensitizing *EGFR* mutations eventually predicting TKI sensitivity [50]. Accordingly, in cell lines p.A763_Y764insFQEA showed sensitivity at lower concentrations than 0.1 μmol/L to erlotinib, in the very low micromolar range to afatinib and osimertinib whereas retaining slight sensitivity only to gefitinib [32]. Thus, p.A763_Y764insFQEA appeared to display sensitivity to all approved EGFR TKIs in the preclinical setting and to be associated with improved clinical outcomes following treatment with only second-line erlotinib (median PFS of 17.4 months, median OS of 24 months) [31], claiming for more robust clinical first-line data to establish the best treatment choice even when compared to chemotherapy-based regimens. p.A767_V769dupASV was associated with resistance to first-generation EGFR TKIs in both preclinical [28] and clinical setting [51,52] while showing a wide therapeutic window for afatinib and osimertinib in cell lines studies [51] as well as a lack of response to afatinib in a xenograft assay [47]. Likewise, despite the need for more solid *in vivo* data, *in vitro* findings confirmed that p.V769_D770insASV was not even sensitive to high doses of first-generation TKIs (> 3 μmol/L) while demonstrating promising activity especially for afatinib (< 0.1 μmol/L) comparing to osimertinib (0.333 μmol/L) [32]; strikingly, osimertinib showed an interesting tumor growth inhibition to a greater degree than afatinib in certain *in vivo* xenograft models [53], though a similar slight activity for both afatinib and osimertinib (=0.1 μmol/L) was observed in other xenograft models assays expressing the p.V769_D770insASV mutation [27]. Both preclinical and clinical data seemed to confirm p.D770_N771insSVD as a resistant mutation to first-generation TKIs, even if associated with short PFS and longer overall survival rates have been shown in two patients in the SATURN study [31]; notably, in spite of the absence of clinical evidences in literature, afatinib and osimertinib showed only slight activity [27,32]. Nevertheless, currently missing clinical data are warranted to prospectively investigate the efficacy of all generation TKIs in patients harboring uncommon exon 20 insertions, even if some phase II trials evaluating the role of osimertinib are ongoing (NCT03191149; NCT13414814).

Regarding exon 20 point mutations, the single p.S768I mutation represents approximately 1% of all *EGFR* mutations, even if often existing as compound mutations [54]. Although the actual frequency of the complex mutation remains unclear with the single mutation experiencing inferior outcomes compared to tumors with compound p.S768I mutations [55], the majority of available clinical findings reported responses to TKIs in tumors with complex p.S768I mutations. As a matter of fact, the combined post-hoc analysis of LUX-lung 2, 3 and 6 trials reported excellent objective responses and longest survival rates (median PFS of 14.7 months) for patients with p.S768I mutations treated with afatinib, even if seven of eight patients presented with complex mutations [13]. As for first-generation TKIs, preclinical findings seemed to resemble the relative resistance of this point mutation to erlotinib and gefitinib [24,27,32], consequently resulting in inferior clinical reported outcomes in most retrospective series [56,57]. Furthermore, *in vitro* and xenograft studies were consistent with clinical reports observed in patients harboring the p.S768I mutation, demonstrating the superiority of afatinib (IC50 = 0.0007 μmol/L) when compared to first-generation TKIs and, to a lesser extent, to osimertinib which retained a satisfactory sensitivity profile (IC50 = 0.049 μmol/L) [27,32]. From the clinical standpoint, in a prospective multicenter phase II trial osimertinib has been only recently associated with a median PFS of 12.3 months in patients with just the p.S768I mutation [58], although further results of other ongoing phase II prospective clinical trials are awaited (NCT03434418).

In addition to the drug-induced selection of a threonine-methionine amino acidic substitution at position 790 (T790M) which is thought to be the most common mechanism of resistance to first-generation TKIs [59], the acquired exon 20p.C797S point mutation represents the most common resistance mechanism to third-generation TKIs [60]. Based on preclinical models, the single p.C797S mutation appeared to confer resistance to most of irreversible TKIs, affecting the cysteine residue at position 797 used by these drugs to covalently bind with the receptor [61,62]. *In vitro* studies seemed to be consistent with the available xenograft models assays, confirming to be associated with complete

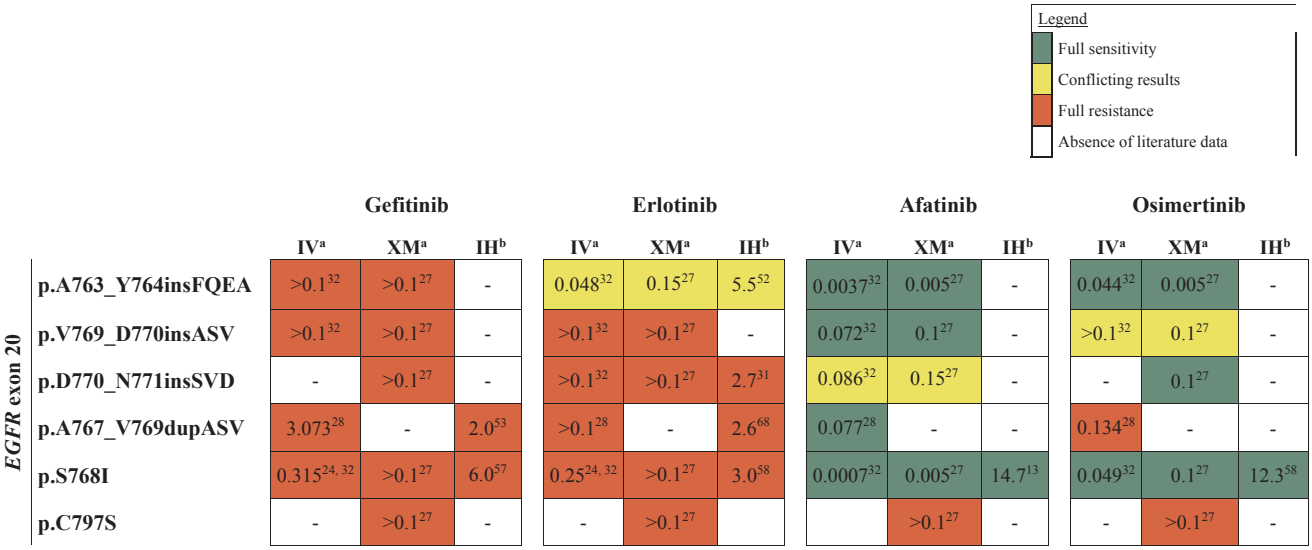

Fig. 4. Overview and assessment of EGFR TKIs activity in cell lines (IV), xenograft models (XM) and patients (IH) harboring exon 20 mutations [[a]expressed in term of micromolar (μM) concentration, [b]expressed in term of median months of progression-free survival (PFS)]. The drug sensitivity was color-coded according to the scheme indicated at the top right and was categorized as sensitive, resistant, controversial or not available based on literature data.

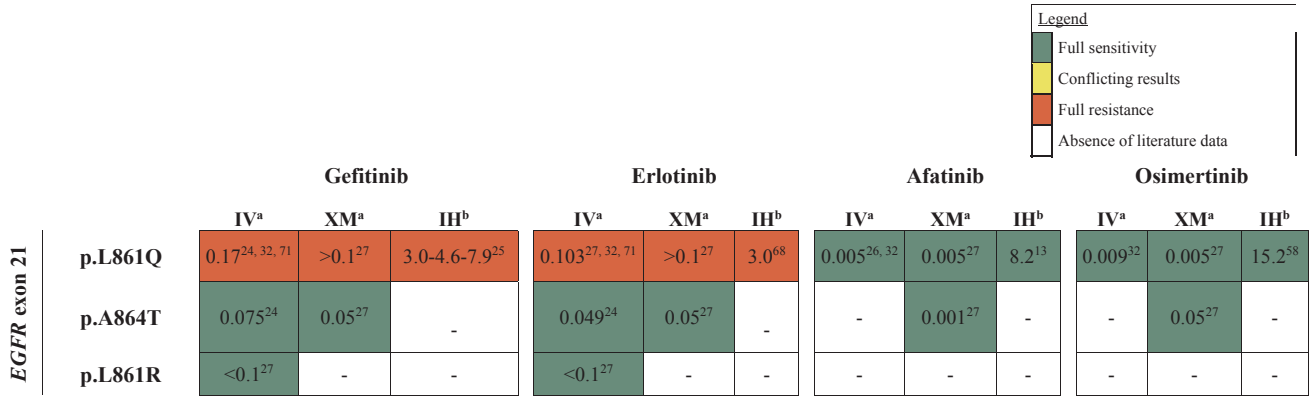

Fig. 5. Overview and assessment of EGFR TKIs activity in cell lines (IV), xenograft models (XM) and patients (IH) harboring exon 21 mutations [[a]expressed in term of micromolar (μM) concentration, [b]expressed in term of median months of progression-free survival (PFS)]. The drug sensitivity was color-coded according to the scheme indicated at the top right and was categorized as sensitive, resistant, controversial or not available based on literature data.

resistance to osimertinib (IC50 = 5 μmol/L) while showing only slight sensitivity to first-generation TKIs and afatinib (IC50 = 0.5 μmol/L) [27]. As suggested by previous *in vitro* evidences showing that first-generation EGFR TKIs seemed to exert their efficacy independent of the cysteine at position 797 [63], it is noteworthy that a combination of first- and third-generation EGFR TKIs resulted to be more clinically effective only when p.C797S and p.T790M mutations have been detected *in trans* [64,65], even if clear clinical outcomes are currently not available. Intriguingly, the definition of *trans* configuration was related to the location of mutations that, however, could exist on different DNA strands occurring either within different alleles of the same clone or in different tumor cell clones, ultimately leading to different clinical scenarios [66]. On the other hand, if mutations were detected *in cis*, the *EGFR* activity appeared to be suppressed neither by the EGFR TKIs alone nor in combination (Fig. 4) [67].

Exon 21

The most commonly identified *EGFR* mutation occurring within exon 21 includes the point mutation which substitutes arginine for Leucine at codon 858 (p.L858R). However, a heterogeneous group of other non-classical *EGFR* point mutations may occur besides L858R and have been collectively associated with decreased sensitivity and poor clinical outcomes to first-generation TKIs in both preclinical and clinical studies [68,69].

The p.L861Q mutation represents the second most frequent mutation occurring within the exon 21, accounting for approximately 1–2% of all *EGFR* mutations and not rarely presenting as complex mutation. Despite preclinical data suggested a decreased sensitivity to gefitinib and erlotinib in both *in vitro* [24] and xenograft studies [27], retrospective data showed that some patients harboring the p.L861Q mutation exhibited a modest response to first-generation EGFR TKI treatment [25]. On the other hand, available clinical data with afatinib appeared to corroborate preclinical findings of broad activity, considering that the *in vitro* sensitivity of this mutation in the low micromolar range showed no significant differences in IC50 values comparing to osimertinib while being, more relevantly, consistent with improved PFS as well as OS rates [13]. Moreover, clinical data investigating the role of osimertinib in such patients have recently showed high response rates along with interesting PFS rates of 15.2 months [58], confirming its *in vitro* efficacy [32]. Other more rarely detected gene variants may occur within the exon 21 and include point mutations that generally appeared to be much more sensitive to afatinib and osimertinib (Fig. 5), even if clinical data are missing and first-generation TKIs were revealed to be slightly active in the prclinical setting (for instance, in p.A864T cell lines [24] and in p.L861R

xenograft models assays) [27].

Complex mutations

Complex EGFR mutations, commonly defined as compound or double or multiple mutations in the *EGFR* tyrosine kinase domain, have been frequently detected by NGS but their clinical value remain ambiguous with variable and not fully elucidated responses to TKIs [70].

The prevalence of detection rate of compound *EGFR* mutations has increased from 4% to 14% over the past years [19,70]. More recently, with the introduction of NGS analysis, the identification of complex mutations was reported in up to 25% of the *EGFR*-mutated population [71], suggesting that the frequency of these mutations is other than insignificant. In most cases these mutations consist of one classical sensitizing mutation together with a rare partner mutation of unknown clinical significance or an atypical uncommon mutation [71]. The sensitivity pattern of the accompanying mutation seemed to significantly affect the efficacy of the TKI in most compound mutations [30].

Recent preclinical evidences seemed to suggest that second-generation TKIs such as afatinib may have a broader inhibitory profile against *EGFR* compound mutations than first- or even third-generation TKIs, even if, as expected, less effective than osimertinib in T790M mutations [27]. Indeed, preclinical evidences suggested the association of gefitinib and erlotinib with significantly diminished inhibitory activity against certain emerging complex mutations (e.g. p.L858R + p.E709A/G/K) which actually resulted to be strongly inhibited in the low micromolar range by afatinib and, less markedly, osimertinib [27]. Moreover, the *in vitro* characterization p.S768I + p.G719A was confirmed in xenograft models assays, resulting to be resistant to first-generation TKIs also in the rare clinical setting [52] and highly sensitive to afatinib, while being only slightly inhibited by osimertinib [27]. Clinical retrospective data investigating the efficacy of gefitinib in NSCLC patients harboring complex mutations seemed to corroborate the preclinical findings, demonstrating a median PFS of 6.0 and 10.0 months in patients with p.L858R + p.S768I and p.L858R + p.E709K, respectively, while even shorter survival rates in patient presenting with p.G719S/A/C mutations in combination with other atypical *EGFR* gene alterations were observed [72]. Among all the uncommon gene alterations, these compound mutations seemed to be associated with improved clinical outcomes, even if a high degree of heterogeneity plays a crucial role in it [71]. Other retrospective patient series suggested that complex mutations containing the classical sensitizing p.L858R or exon 19 deletions showed poorer PFS rates when treated with first-generation TKIs and compared to single mutations (median PFS of 5.3 vs 8.0 months, respectively) [19], whereas in the study by Peng *et al.* no significant differences in PFS and OS rates between NSCLC patients with single mutation involving Leucine in position 858 and in association with other exon 18–21 co-mutations were observed with gefitinib (Table 1) [73].

Discussion

In this review, we summarized and critically discussed *in vitro* and *in vivo* data to provide a comprehensive overview of incidence, sensitivity pattern and outcomes of less frequently detected *EGFR* mutations in both preclinical and clinical setting of advanced non-small cell lung cancer. Despite these populations of *EGFR* mutations have been collectively named “uncommon” to be distinguished from the classical sensitizing mutations with well-described clinical significance, we may consider them as “untested” in the light of the underestimated incidence obtained even with the most commonly FDA-approved Real–Time polymerase chain reaction (PCR)-based assays used in clinical trials [55,74].

Testing of inadequate and low-quality tissue sample as well as the heterogeneity in detection techniques has fatally resulted in inaccuracies and biases in the reported incidence of less common *EGFR* mutations, even in pivotal and practice-changing phase III randomized clinical trials [55]. Indeed, the wide use of PCR-based commercial assays such as Therascreen (Qiagen Manchester, UK) or Cobas (Roche, Basel, Switzerland), which only considered “hot spots” thought predictive of TKI response, have posed significant diagnostic issues of detection sensitivity, even in a recent prospective post-hoc analysis that was precisely specified to evaluate the clinical activity of a second-generation TKI in patients harboring *EGFR* minor mutations [13,75]. For instance, the exon 18 “G719X mutation”, which have been extensively reported in literature in several preclinical and clinical series, actually referred to point mutations, leading to substitutions of the glycine at position 719 to other amino acidic residues (S/A/C) and consequently resulting in different *in vitro* and *in vivo* TKI actionability [26–30]. More recently, given the need for a more comprehensive gene profiling in NSCLC metastatic setting, different NGS gene panels have been implemented into clinical practice, increasing significantly the reference range in term of gene regions coverage and limit of detection while leading to the improvement of *EGFR* mutations detection [76]. However, despite being present in at least 10% of patients with *EGFR* mutation-positive NSCLC because of recent improvements in detection methods, less common mutations have been excluded in most phase III trials, like the FLAURA study, evaluating the clinical efficacy of TKIs in advanced *EGFR*-mutated NSCLC patients [6–10]. Furthermore, even if several randomized clinical trials (LUX-Lung 7, FLAURA, ARCHER-1050) have demonstrated that second- and third-generation TKIs have led to improved PFS rates when compared to first-generation TKIs in a front-line setting, until recently no head-to-head comparisons between the different generations of TKIs have been conducted in patients with less common *EGFR* mutations [77].

When systematically reviewing the present literature, we observed that *in vitro* and xenograft studies seemed to resemble the mixed and mostly retrospective data available regarding the activity of all the EMA-approved EGFR TKIs in tumors harboring less frequently detected activating mutations. Even if clinical data with second- and third-generation TKIs are scant, exon 18p.E709K/A/G/V mutations appeared to be suitable for afatinib whereas p.G719A/C/S point mutations showed varying drug sensitivities and clinical responses to EGFR TKIs, depending on the precise amino acid substitution; in particular, p.G719C appeared to be sensitive to any type of EGFR TKIs whereas p.G719A was strongly inhibited by afatinib, thereby underlining the strong importance of very specific techniques in the diagnostic setting. Activating indel alterations within exon 19 appeared to be resistant to first-generation TKIs while retaining a remarked sensitivity to both afatinib and osimertinib. Exon 20 insertions confirmed their wide variability, showing interesting preclinical activity in favor of afatinib and osimertinib in some specific cases that, however, still need to be validated in ongoing prospective clinical trial. The most prevalent exon 20 mutations, including insertions and point mutations, were confirmed to be resistant to first-generation TKIs while afatinib and osimertinib showed promising preclinical activity, especially for p.A763_Y764insFQEA. As concerns exon 21, the p.L861Q point mutation exhibited a modest response to first-generation EGFR TKI treatment while showing remarkable preclinical and clinical prospective data in favor of afatinib and osimertinib. Regarding compound mutations, the insufficient and anecdotal available data seemed to be controversial, suggesting that the sensitivity pattern of the accompanying mutation seemed to significantly influence the TKI efficacy. However, afatinib (and to a lesser extent osimertinib) retained a strong preclinical activity against certain emerging complex mutations such as p.L858R + p.E709A/G/K or p.S768I + p.G719A. The *trans* and *cis* configurations of compound mutations, especially for the p.C797S mutation, has resulted to be another relevant point of discussion leading to different patterns of sensitivity and resistance, respectively, since co-existing *in cis* mutations likely appeared to have an additive impact on the tertiary structure of *EGFR* underlying their role in terms of acquired resistance [77]. In

Table 1
Overview of EGFR TKIs activity in cell lines (IV), xenograft models (XM) and patients (IH) harboring complex mutations.

EGFR exon	Nucleotide variant	Protein change	Gefitinib			Erlotinib			Afatinib			Osimertinib		
			IV[a]	XM[a]	IH[b]	IV[a]	XM[a]	IH[b]	IV[a]	XM[a]	IH[b]	IV[a]	XM[a]	IH[b]
18	c.2126A > C	p.E709A	0.032	0.0001[27]	–	–	0.0001[27]	12.0[31]	–	0.0001[27]	–	–	0.0005[27]	–
+	+	+	–											
18	c.2155G > T	p.G719C	0.512[30]											
18	c.2126A > C	p.E709A		0.05[27]	–	–	0.05[27]	8.0[72]-8.3[31]	–	0.0001[27]	–	–	0.05[27]	–
+	+	+												
18	c.2155G > A	p.G719S												
18	c.2155G > A	p.G719S	–	–	32.0[25]	0.1–0.05[26]	–	–	0.005[26]	–	–	–	–	–
+	+	+												
21	c.2582 T > A	p.L861Q												
18	c.2126A > C	p.E709A	–	> 0.1[27]	–	–	0.1[27]	–	–	0.0005[27]	–	–	0.005[27]	–
+	+	+												
21	c.2573 T > G	p.L858R												
18	c.2126A > C		–	0.1[27]	–	–	0.05[27]	–	–	0.0005[27]	–	–	0.01[27]	–
+	+	p.E709A												
21	c.2573 T > G	+												
		p.L858R												
18	c.2126A > G +		–	0.05[27]	–	–	0.05[27]	–	–	0.0005[27]	–	–	0.0001[27]	–
+	c.2573 T > G	p.E709G												
21		+												
		p.L858R												
18	c.2125G > A + c.2573 T > G	p.E709K	–	0.05[27]		–	0.05[27]	–	–	0.0005[27]	–	–	0.0001[27]	–
+		+			10.0[75]									
21		p.L858R												
20	c.2303G > T	p.S768I	–	> 0.1[27]	1.2[53]	–	> 0.1[27]	5.0–7.0[72]	–	0.0005[27]	–	–	0.1[27]	–
+	+	+												
18	c.2156G > C	p.G719A												
20	c.2303G > T	p.S768I												
+	+	+	–	0.01[27]	–	–	0.0005[27]	–	–	0.0001[27]	–	–	0.001[27]	–
18	c.2155G > T	p.G719C												
20	c.2303G > T	p.S768I	–	0.05[27]	–	–	0.01[27]	–	–	0.0005[27]	–	–	0.05[27]	–
+	+	+												
18	c.2155G > T	p.G719C												
20	c.2303G > T	p.S768I	–	0.05[27]	–	–	0.005[27]	–	–	0.0001[27]	–	–	0.05[27]	–
+	+	+												
18	c.2155G > A	p.G719S												
20	c.2303G > T	p.S768I	–	0.1[27]	–	–	> 0.1[27]	–	–	0.0005[27]	–	–	> 0.1[27]	–
+	+	+												
18	c.2155G > A	p.G719S												
20	c.2369C > T	p.T790M	–	> 0.1[27]	–	–	> 0.1[27]	–	–	> 0.1[27]	–	–	> 0.1[27]	–
+	+	+												
20	c.2390G > C	p.C797S												
20	c.2303G > T	p.S768I	–	0.1[27]	6.0[75]-21.7[53]	–	0.05[27]	–	–	0.0005[27]	–	–	0.0005[27]	–
+	+	+												
21	c.2573 T > G	p.L858R												
21	c.2582 T > A	p.L861Q	–	0.1[53]	–		0.05[53]	–	0.005[26]	0.0005[53]	–	–	0.005[53]	–
+	+	+				0.1–0.05[26]								
18	c.2156G > C	p.G719A												
21	c.2582 T > A	p.L861Q	–	0.01[27]	–	–	0.005[27]	–	–	0.0001[27]	–	–	0.0001[27]	–
+	+	+												
21	c.2573 T > G	p.L858R												

(*continued on next page*)

Table 1 (*continued*)

EGFR exon	Nucleotide variant	Protein change	Gefitinib			Erlotinib			Afatinib			Osimertinib		
			IV[a]	XM[a]	IH[b]	IV[a]	XM[a]	IH[b]	IV[a]	XM[a]	IH[b]	IV[a]	XM[a]	IH[b]
21	c.2582 T > A	p.L861Q	–	> 0.1[27]	–	–	0.1[27]	–	–	0.0005[27]	–	–	0.005[27]	–
+	+	+												
21	c.2573 T > G	p.L858R												

[a] Expressed in term of micromolar (μM) concentration.
[b] Expressed in term of median months of progression-free survival (PFS)].

summary, considering the high molecular heterogeneity, the relative paucity of clinical data and the lack of formal statistical comparisons, such observations might be valuable options for clinical practice, strongly encouraging the implementation of a NGS-based approach to specifically characterize the *EGFR* mutational status in NSCLC patients [76,78].

In this fascinating and very complex landscape and in absence of head-to-head prospective randomized data, the role of such observations reported in this review, entrusted with the management of a multidisciplinary team (Molecular Tumor Board), may represent the key weapon to better define and optimize the treatment strategy for NSCLC patients harboring less frequently detected *EGFR* mutations.

Funding

The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

CRediT authorship contribution statement

Valerio Gristina: Conceptualization, Data curation, Formal analysis, Investigation, Software, Writing - original draft. **Umberto Malapelle:** Conceptualization, Investigation, Supervision, Visualization, Writing - review & editing. **Antonio Galvano:** Writing - review & editing, Formal analysis, Investigation, Methodology, Software, Writing - original draft. **Pasquale Pisapia:** Writing - original draft, Investigation, Software. **Francesco Pepe:** Writing - original draft, Investigation, Software, Writing - original draft. **Christian Rolfo:** Supervision, Validation, Visualization, Writing - review & editing. **Silvia Tortorici:** Supervision, Validation, Visualization, Writing - review & editing. **Viviana Bazan:** Methodology, Supervision, Validation, Writing - review & editing. **Giancarlo Troncone:** Conceptualization, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing. **Antonio Russo:** Conceptualization, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing.

Declaration of Competing Interest

Dr. Malapelle reports personal fees from Boehringer Ingelheim, AstraZeneca, Roche, MSD, Amgen, Merck, BMS for participation in a speaker bureau; personal fees from Boehringer Ingelheim, MSD, Amgen, Merck, BMS for acting in an advisory role, financial support from Boehringer Ingelheim and Amgen that was paid directly to his institution outside the submitted work. Prof. Rolfo reports personal fees from Merck Sharp and Dohme, Astrazeneca, for participation in a speaker bureau; personal fees from Mylan, Archer, Merck Serono, Inivata for acting in an advisory scientific role; and has research collaborations with Biomarkers and Guardant Health outside the submitted work. Prof. Troncone reports personal fees from Roche for participation in a speaker bureau; personal fees from MSD, Pfizer for acting in an advisory role. The remaining authors declare no potential conflicts of interest.

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