NSCLC Concurrent EGFR Genomic Alterations

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Non-small cell lung cancer (NSCLC) accounts for roughly 85–90% of overall cases of lung malignancies and includes different histological subtypes. The treatment landscape of NSCLC has been terrifically changed by the discovery of Epidermal Growth Factor Receptor (EGFR) mutations and their response to the EGFR tyrosine kinase inhibitors (TKIs). EGFR gene aberrations have been defined as oncogenic driver mutations which occurred in 5–17% of lung adenocarcinomas among Caucasian patients, while in approximately 45–55% of the Asian population. Nowadays, EGFR-TKIs are the standard of care for patients affected by advanced EGFR-mutated NSCLC considering their established prolonged progression-free survival (PFS) in comparison to the standard chemotherapy approach. However, TKIs clinical efficacy remains restricted due to the development of resistance, which has been hardly clarified. The recent technological breakthrough and the advent of next-generation sequencing (NGS) platforms have enabled comprehensive profiling of the genome, providing novel evidence of co-existing multiple driver alterations.

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1. Complex EGFR Mutations

Of note, almost 45% of *EGFR* gene aberrations are in-frame deletion alterations in exon 19 (19Del) and the p.L858R within exon 21 [1][2]. These activating mutations enhance a better outcome in patients, granting a complete blockade of the *EGFR* signaling pathway by EGFR-TKIs. Otherwise, *EGFR* mutations occurring in exons 18 and 20 are correlated with resistance to standard treatments. Uncommonly, complex *EGFR* alterations could be detected in a single tumor specimen harboring two or more various intra-*EGFR* mutations [3]. Complex *EGFR* mutations occur almost in 3–7% of *EGFR*-mutant patients [4]. Belardinilli et al. described a single clinical case of an NSCLC patient harboring three coexisting aberrations on the *EGFR* gene, two of which presented on the same allele [2]. In fact, through the use of NGS, the authors detected the simultaneous presence of three missense mutations, a p.L858R and p.L861R both in exon 21 with an allele frequency close to 41%, and a p.R776H in exon 20 with an allele frequency of 67.5%, respectively. Besides, upon therapy with the second-generation EGFR-TKI afatinib, the patient showed a partial response on the target lung lesion with a PFS of eight months. Moreover, a clinical trial conducted by Lee et al. investigated molecular backgrounds of primary resistance to EGFR-TKIs in NSCLC patients harboring sensitive *EGFR* alterations [5]. The study population included a cohort of 197 patients, out of whom nine individuals had two co-existing *EGFR* mutations. Additionally, among 11 patients exhibiting de novo resistance to TKI treatment only one patient had a coexisting *EGFR* complex mutation, particularly p.T790M mutation and 19Del. The authors reported that this patient displayed immediate disease progression involving symptomatic metastasis to the central nervous system (CNS) while receiving EGFR-TKI

treatment. Furthermore, a recent analysis by Liang et al. evaluated concomitant alterations in *EGFR* 19Del/L858R mutation and their correlation with EGFR-TKIs response in a total of 403 NSCLC patients [6]. This trial included two cohorts and comprehensively analyzed the concomitant mutational profiles of *EGFR* 19Del and p.L858R in TKI naïve patients. The authors assessed that the existence of somatic p.T790M at baseline was similar in 19Del (120, 73.4%) and p.L858R (160, 72.4%) mutations. Furthermore, Zhang et al. screened 187 patients with complex *EGFR* mutations out of 5898 *EGFR*-positive NSCLC patients. Fifty-one of these patients were under first-line treatment with first-generation EGFR-TKIs [4]. Namely, 58 patients were found to carry a concurrent alteration in *EGFR* exon 20 and 21, while 45 patients harbored a concomitant mutation in exon 19 and 21. Considering the genetic aberrations, simultaneous p.T790M and p.L858R were the most common, followed by 19Del and p.L858R. The median PFS was 9.5 months. The overall response rate (ORR) was 52.2% (95% CI 37.2–67.2%), and the disease control rate (DCR) was 71.7% (95% CI, 58.2–85.3%). Additionally, the authors subdivided patients into four groups: A) patients with 19Del and p.L858R; B) patients harboring a 19Del or p.L858R and atypical mutations; C) double atypical mutations; and D) complex mutations with a primary drug-resistant pattern, such as a primary p.T790M mutation or an exon 20 insertion. As reported by the authors, NSCLC patients with exon 19Del and p.L858R exhibited the best ORR and PFS, 75% and 18.2 months, respectively. On the other hand, patients included in group D displaying complex mutations with a primary drug-resistant pattern, such as a primary p.T790M mutation or an exon 20 insertion, have the worst clinical outcomes. Notably, some of these patients carried a sensitizing *EGFR* alteration (i.e., 19Del/p.L858R/p.L861Q) plus a p.T790M de novo or an exon 20 insertion. Thus, the worst clinical outcomes achieved by these patients could be explained by the fact that they were treated with first and second-generation EGFR-TKIs. Moreover, Benesova et al. described a single case of a patient with complex *EGFR* alteration [7]. Of note, the patient exhibited partial response under treatment with gefitinib. Otherwise, Sato et al. reported that 6 patients with double *EGFR* alterations showed a poorer response to gefitinib treatment [8]. De Marchi et al. found 33 patients with double *EGFR* genomic aberrations in a cohort of 1006 lung cancer patients, with no data being unfortunately available on their clinical outcomes [9]. Li et al. detected 58/5125 *EGFR* double mutations, with the highest incidence rate of p.T790M and p.L858R [10]. Chen et al. presented 4/36 patients harboring concurrent 19del and p.L858R with a worse response after TKI treatment [11]. Additionally, Chen et al. reported concurrent *EGFR* complex genomic alterations in 20 patients with the worst outcome in terms of OS [12].

2. Actionable Concomitant Oncogenic Driver Mutations

Although actionable oncogenic gene driver mutations in NSCLC were historically considered mutually exclusive, the recent advent of comprehensive genomic profiling in clinical specimens was able to identify a notable number of concurrent alterations in *EGFR*-mutated NSCLC. Recently, various original research articles and case reports were conducted on this topic, suggesting that some *EGFR*-mutant NSCLC patients may carry concomitant genetic aberrations in different oncogenic driver genes.

2.1. *ALK*

ALK is a component of the insulin receptor protein-tyrosine kinase superfamily, formerly reported as a nucleophosmin *(NPM)-ALK* fusion pattern in cell lines of anaplastic large cell lymphoma (ALCL) [13]. In 2007 *ALK* fusion was described in lung adenocarcinoma for the first time in a limited cohort of Asian individuals [14]. The most common aberration is an inter-chromosomal inversion in the short arm of chromosome 2, which generates a fusion between the *echinoderm microtubule-associated protein like-4* (*EML4*) gene and the *ALK* gene [15]. Consequently, the fusion *EML4-ALK* with tyrosine kinase function stimulates proliferation and cell survival [15]. Chromosomal rearrangements in the *ALK* gene are detected in approximately 5% of NSCLC patients [16]. Moreover, this driver fusion is predominantly estimated mutually exclusive with other genetic mutations, such as *EGFR* [17]. Notwithstanding, with the advent of novel and powerful technologies like NGS the detection rate of concomitant genetic alterations in *EGFR* and *ALK* is systematically increased [17][18]. Liu et al. evaluated the efficacy of TKI treatments on 21 co-altered *EGFR* and *ALK* patients with advanced NSCLC [18]. Three out of 21 patients received dual blockade TKI treatment with EGFR- and ALK-TKIs, reaching a PFS of 5.2 months with the combination therapy. Furthermore, analyzing the clinical-pathological features of the concomitant mutation patients the authors found that the double genetic alteration was more likely to occur in young females than in males. Additionally, Hu et al. examined the frequency of concurrent genetic alterations in *EGFR*-positive patients, evaluating the efficacy of EGFR-TKIs treatment in this setting [19]. Out of 320 patients including in the study population, six patients were found harboring a co-alteration in *ALK* gene and they achieved a mPFS of five months, shorter compared to those with a single *EGFR* mutation (mPFS 10.9 months). Namely, four out of six patients with concomitant *ALK* rearrangement were treated with the first-generation ALK-TKI crizotinib and three obtained partial response according to RECIST criteria. Considering the particular subset of patients, a recent report by Zhuang et al. determined that ALK-TKI therapy for the treatment of 20 patients with a co-alteration in *ALK* fusion was more active as first-line treatment than in later lines of treatment [20]. Yang et al. assessed that 13/977 NSCLC patients screened harbored a concomitant genetic aberration in *EGFR* and *ALK* genes [21]. Out of 13 patients, 10 naïve patients received EGFR-TKIs reaching an ORR of 80% and a mPFS of 11.2 months (95%CI 5.6–16.8). Four patients were treated with crizotinib, and three of them in a second-line setting. Considering the clinical outcomes, two patients appeared to respond to EGFR-TKI, yet not to ALK-TKI; whereas one was sensitive to crizotinib. The only patient who received crizotinib as first-line displayed 15.1 months of PFS, still not show response to consecutive EGFR-TKI treatment. Patients with *EGFR* and *ALK* coexisting aberrations seemed to better respond to EGFR-TKIs in the first-line setting. Of note, in order to explain the great heterogeneity of clinical outcomes, the authors suggested that different sensitivities to therapies might be correlated with different levels of *EGFR* or *ALK* protein phosphorylation. Fan et al. described a single case of a patient harboring *EGFR/ALK* alteration, who had partial response under ALK-TKI [22]. Besides, Lee et al. described 12 patients with double *EGFR/ALK* alteration, 11 of which with a partial response to treatments based on gefitinib, erlotinib or crizotinib [23]. Notably, Miyanaga et al. described a single case where the patient showed response both to first-generation EGFR-TKIs and crizotinib [24]. Sweis et al. presented a case series including four patients treated with erlotinib and crizotinib, achieving a stable disease as the best response [25]. Thumallapally et al. reported a single case harboring an *ALK* translocation together with an *EGFR* p.L861Q mutation treated with crizotinib reaching a PFS of 3 weeks [26]. In their exploratory study, Lee et al. found two out of 197 *EGFR*-positive NSCLC patients with a concurrent genomic alteration in *ALK* [5]. Notably, the patients were treated with gefitinib and

consequently with crizotinib, achieving a partial response. Chang et al. did not report the clinical outcome of their single case [27], as well as Zhu et al. who described two patients out of 139 [28]. Chen et al. described a single case of double *EGFR/ALK* alteration with poor outcomes [12].

2.2. *KRAS*

KRAS alterations are frequently represented by missense mutations occurring in lung adenocarcinomas [29]. Molecular evaluation of *KRAS* is crucial to predict clinical outcomes and to choose the best therapeutic option, as *KRAS*-mutant tumors exhibit primary resistance to EGFR-TKIs [29]. Moreover, almost 6–35% of *EGFR* positive patients harbor a concomitant genetic aberration in the *KRAS* gene [30]. P.G12C, p.G12V, and p.G12D mutation are the most frequent alteration detected [31]. Several cases have been reported for *EGFR* and *KRAS* concurrent alterations. Benesova et al. presented three cases of patients with *EGFR* mutations combined with *KRAS* mutation [7]. Despite an initial positive response to EGFR-TKI, the real activity did not last long showing a PFS of three, five, and seven months, respectively. Opposing this report, Zhuang et al. reported a retrospective study involving 3774 patients with concurrent genetic alterations [20]. Namely, 11 patients of the cohort showed a co-alteration in *EGFR/KRAS* and they were treated EGFR-TKI therapy as first-line treatment, displaying an ORR of 62.5% (5/8). Interestingly, the PFS comparisons between patients with an *EGFR/KRAS* co-mutation and those carrying a single *EGFR* mutation were not statistically significant. Ranchiglio et al. identified 14 patients with concurrent *EGFR* and *KRAS* mutations, among six with a dominant VAF [32]. Notably, their PFS was significantly shorter compared to *EGFR* mutations (2.42 months vs. 11.09 months; $p = 0.0081$), and also the ORR was poorer (16.7% vs. 57.1%). Additionally, Nardo et al. analyzed the prevalence of concurrent *KRAS* mutations on 106 patients with *EGFR*-mutant NSCLC focusing on their impact on clinical outcome [33]. Indeed, *KRAS* co-alterations were detected in 3 patients with a VAF of less than 0.2%, which showed poor clinical outcome to first-line EGFR-TKI, in terms of time to treatment failure (TTF), OS and PFS (five, six and five months, respectively). Lee et al. described six patients with *EGFR/KRAS* aberration, not reporting their clinical outcomes [23], as Li et al. who reported 30 patients with double alterations out of a cohort of 5125 individuals [10]. Chevallier et al. described a single case [34], as De Marchi et al. [9]. Moreover, Zhang et al. found two out of 120 patients with double concurrent genomic aberrations [4]. Whereas Hu et al. described a single case of *EGFR/KRAS* out of a cohort including 320 individuals [19], of note the patient showed progression after treatment with erlotinib. Finally, in the trial by Chen et al. [12], seven out of 36 patients displayed a concurrent alteration in *EGFR* and *ALK* with poorer PFS after EGFR-TKI treatment.

2.3. *ROS-1*

ROS-1 rearrangements has been detected in almost 1–2% of lung adenocarcinoma [35]. The ALK-TKI crizotinib is highly active in *ROS1*-rearranged patients [36]. Patients harboring a concomitant mutation in *EGFR/ROS-1* are very rare, thus we found little data in the current literature. Zhu et al. described a case of a single patient with concurrent *EGFR/ROS-1* alteration [35]. Moreover, in the above-mentioned article by Zhuang et al., two out of 3774 patients harbored a co-alteration in *EGFR/KRAS/ROS-1*. Namely, one patient showed a progression after second-line treatment with crizotinib and partial response to icotinib as third-line treatment (PFS of 27.5 months), while the

second patient had a partial response after first-line treatment with gefitinib (PFS of 12.7 months) [20]. Hu et al. reported one out of 320 patients with double *ROS-1/EGFR* genomic alteration and a partial response after erlotinib as first-line treatment [11].

2.4. *MET*

Mesenchymal–epithelial transition (*MET*) encodes a transmembrane tyrosine kinase, which activates downstream signaling pathways by binding to the hepatocyte growth factor. Thusly, it has a crucial role in cell proliferation and survival [37]. *MET* alterations are emerging as important driver aberrations for NSCLCs, particularly *MET* gene amplification and exon 14 skipping mutations are found with a frequency of 1–11% and up to 4% in lung adenocarcinoma [38]. *MET* amplification is a well-known resistance mechanism against EGFR-TKIs, including the third-generation osimertinib [39][40]. Indeed, *MET* amplification is accountable for almost 5–22% of secondary resistance to EGFR-TKIs. Particularly, *MET* amplification induces ErbB3 phosphorylation, hence activating the *PI3K/AKT* pathway [38]. In line with these data, the treatment combination of EGFR-TKIs and MET-inhibitors has been evaluated in different clinical trials, such as INSIGHT 1 and TATTON [41][42]. Namely, in the phase 1b/2 clinical trial INSIGHT 1, Wu et al. and colleagues evaluated the efficacy of the combination tepotinib/gefitinib in *EGFR*-mutant patients with *MET* amplification and secondary resistance to EGFR-TKIs, reporting better mPFS and mOS in this particular subset of patients (16.6 vs. 4.2, HR 0.13; 37.3 vs. 13.1 HR 0.08, respectively) [41]. Additionally, Oxnard et al. examined the safety of osimertinib in combination with selumetinib/savolitinib/durvalumab [40]. Indeed, only three patients harbored *MET* amplification and p.T790M and they were treated with selumetinib displaying partial response [40]. However, osimertinib combination with savolitinib in patients with *MET*-driven secondary resistance to EGFR-TKIs is under current evaluation in the ongoing trials SAVANNAH (NCT03778229) and ORCHARD (NCT03944772). Whereas *MET* exon 14 skipping/*EGFR* mutations are very rare and poorly explored. In preclinical models *MET* ex14 decrease sensitivity to EGFR-TKIs [42]. As results of our systematic review of the literature, we found only three papers presenting interesting data on this particular setting. In fact, Chevallier et al. reported 15 patients with *EGFR/MET* alteration known to be non-pathogenic according to international database [34]. Lee et al. described a single patient with *MET* amplification >15 gene copies in 17% of tumor cells [5]. Chen et al. reported a single case including in the short PFS group (10% vs. 33% $p = 0.018$) [12]. Finally, there is a strong rationale for the use of combination of EGFR-TKIs and MET inhibitors in this setting, thus larger studies are warranted.

2.5. *BRAF*

BRAF mutations, both p.V600E and non-p.V600E, are detected in 6–8% of NSCLC cases, inducing downstream activation of the MAPK signaling pathway [43]. Over the decades, several BRAF inhibitors have been developed and the combination of trametinib and dabrafenib was the first treatment approved for advanced BRAF p.V600E-mutant NSCLC [44][45]. Concomitant *EGFR/BRAF* aberrations are found in approximately 11% of EGFR-positive NSCLC patients, with the *BRAF* p.V600E mutation most frequently identified [46][47]. Chen et al. retrospectively screened 423 NSCLC patients harboring *EGFR* 19Del or p.L858R mutations reporting only one patient with concurrent *BRAF* p.V600E [12]. Of note, the patient showed a poor PFS. Furthermore, Li et al. assessed a

comprehensive mutation profiling from 5125 Chinese cohorts and they reported 160 concurrent mutations including two *EGFR/BRAF* concomitant mutations [10]. Moreover, Rachiglio et al. found hotspot mutation in several genes, including *BRAF* in 14 patients (21.8%) of their cohort [32]. Zhuang et al. described two cases of concomitant *EGFR/BRAF* alteration, showing better outcomes with EGFR-TKI than with standard chemotherapy [20].

2.6. *RET*

Rearranged during transfection (*RET*) gene rearrangements are detected in almost 1% of NSCLC patients [48][49]. Recently, FDA has granted accelerated approval to pralsetinib and selpercatinib for lung cancer patients harboring *RET* fusion based on ARROW and LIBRETTO-001 clinical trials results [50][51]. In up to 10% of NSCLC patients under osimertinib treatment, oncogenic fusions of *RET* gene have been considered responsible for acquired resistance [50][52]. Taking into account this, the open-label, multicenter, biomarker-guided, phase 2 clinical trial ORCHARD (NCT03944772) is still recruiting NSCLC patients progressed on 1-L osimertinib therapy, and one cohort includes *RET* rearranged patients which will receive osimertinib in combination with selpercatinib (LOXO-292) [53][54]. Albeit, the co-presence of *EGFR* mutation and *RET* rearrangement is rare, we found a single case report and a research article presenting original data on this particular subset of patients. Hu et al. detected one patient with concurrent *EGFR* and *RET* genomic alteration out of a cohort including 320 *EGFR* positive patients [19]. Particularly, the patient was an Asian young female with lung adenocarcinoma with no history of smoking, treated with gefitinib displaying poor OS and PFS (10.2 and 2.2 months, respectively) and PD as best response. Moreover, Klempner et al. and colleagues reported two patients with secondary acquired *RET* fusion in Asian *EGFR*-mutant NSCLC patients, both presenting short survival [55]. Of note, none of the patients reported underwent a combination treatment with EGFR-TKIs and RET-inhibitors. These data available from the literature confirmed the fact that *RET* fusion is a resistance mechanism in *EGFR* mutated patients and larger clinical trials are warranted in order to evaluate the potential activity of the combo EGFR-TKIs and RET-inhibitors.

3. TP53, PTEN, PIK3CA, CDKN2A and RB1

TP53 gene mutations are identified in 35–55% of NSCLC cases, especially in squamous cell carcinoma (SCC) and in smokers or former smokers [56][57][58]. Inactivating mutations of the *TP53* gene affect the normal transcriptional p53 activity leading to tumor susceptibility and hinder patients' response to chemotherapy treatments [59][60]. Moreover, *TP53* alterations might be related to a poor prognosis in NSCLC patients [61]. Almost 55–65% of *EGFR*-positive NSCLC patients harbor a *TP53* coexisting mutation [5][62][63]. Preclinical models have already demonstrated a correlation between *TP53* mutation and response to EGFR-TKIs therapy [63][64][65]; namely apoptosis induced by gefitinib is decreased in p53 mutated cells. Mutation in *TP53* gene have been divided into disruptive mutations and non-disruptive ones considering the loss of function of p53 protein. Specifically, disruptive mutations produce a complete loss of function of p53, while non-disruptive alterations result in conservative mutations or non-conservative mutations (excepting stop codons) outside the L2–L3 region [64][66][67][68]. Comprehensively, the systematic literature review identified a total of 11 reports evaluating the *TP53* status in *EGFR*-mutant patients with lung adenocarcinoma. Namely, Canale et al. conducted an independent

retrospective cohort study on a total of 136 *EGFR*-mutated NSCLC patients under treatment with first or second-generation TKIs as a first line therapy, in order to assess the role of *TP53* gene alterations as predictor of survival and response to EGFR-TKIs therapy [69]. Endpoints of the clinical study were DCR, ORR, PFS and OS. TP53 mutations were detected in 42 (30.9%) out of the 136 patients, indeed according to the classification of *TP53* aberrations into disruptive and non-disruptive mutations, the authors observed 11 patients harboring a disruptive *TP53* mutation, while most of the patients carried a non-disruptive alteration [68][70]. Thusly, the authors found that *TP53* mutations in exon 8 are related to a worse PFS regardless to the EGFR-TKIs treatment. Moreover, after a combined analysis the authors confirmed that the worse clinical outcome was independent from the subtype of *EGFR* mutations reported. Of note, further analysis was conducted on a sub-cohort of lung adenocarcinoma patients who developed a p.T790M resistance mutation and treated with osimertinib. This broadened analysis confirmed worse PFS and OS. These data were consistent with a previous report by Hou et al. [71]. In fact, this clinical trial examined the impact of *TP53* gene alterations on the clinical outcomes in a Chinese cohort of 163 patients with NSCLC. By using NGS to establish the mutational status of *EGFR* and *TP53*, 43 *EGFR*-positive patients were found harboring a concurrent *TP53* gene alteration. Considering the treatment outcomes, this subset of patients showed shorter median PFS (6.5 vs. 14.0 months) and median OS (28.0 vs. 52.0 months). Notably, differences in outcomes were particularly meaningful in the subset of patients harboring *TP53* gene non-missense mutations, non-disruptive mutations, mutations in exon 6 and in exon 7 and mutations in the non-DNA Binding Domain (DBD) region among all *TP53* mutations. Interestingly, these data are consistent with the report by VanderLaan et al. [72] who described 10 patients with *TP53* concurrent mutation and worse clinical outcomes. Of note, the authors demonstrated a decreased rate of acquired p.T790M mutation as a mechanism of resistance to gefitinib, erlotinib and afatinib in lung adenocarcinomas with concomitant *TP53* mutations. This could be explained as genomic complex tumors might trigger different pathways bypassing *EGFR* as a target. Additionally, an intriguing retrospective research was reported by Chen et al., who validate the number of concurrent mutation and Tumor Mutational Burden (TMB) in 71 patients with *EGFR* mutation and under treatment with EGFR-TKIs stratified for PSF [12]. Namely, TMB was defined as somatic, coding, base substitution, and indel mutations per megabase of genome analyzed. No significant differences were assessed between the two groups, yet the shorter PFS subgroup revealed a TMB higher than eight. One could guess that an increased TMB is correlated with the existence of resistance pathways, as previous reports suggested [67]. Furthermore, among overall clinical studies, EGFR-TKIs appeared to have less activity in 67 patients harboring concomitant *TP53* gene mutations. A novel treatment option for this particular subset of patients is represented by the combination of EGFR-TKIs and antiangiogenic agents. Indeed, the combination of anlotinib plus icotinib displayed promising activity in the ALTER-L004 clinical trial for *EGFR*-positive NSCLC patients. Namely, the intention to treat population (ITT) included 14 patients carrying concomitant *TP53* alterations, which showed ORR of 78.5% and DCR of 100% [73]. Additionally, in the ACTIVE study, Zhang et al. reported better PFS in the apatinib plus gefitinib group in naïve patients with *EGFR* mutations and patients harboring *TP53* exon 8 mutations showed significant benefit from the dual blockade (HR 0.24 95%CI 0.06–0.91) [74][75]. Rachiglio et al. described 23 *EGFR/TP53* mutant cases, exhibiting a mPFS of 12.3 months and mOS of 18.9 months under EGFR-TKI treatment [32]. Interestingly, Sato et al. reported 12 patients (28%) with *EGFR/TP53* alteration [8]. Moreover, Zheng et al. demonstrated that 11 patients with co-existing *EGFR* and *TP53* genomic alteration might have a worse prognosis comparing to *EGFR*-mutant patients

[76]. Lee et al. described three cases out of 197 patients [5]. Chevallier et al. reported 15 cases of double mutation, with no difference of survival [34]. Chang et al. found that *TP53* was the most common concomitant alteration detected (10/31 patients) [27], as Chen et al. reported in their study [12].

Phosphatase and tensin homologue deleted on chromosome 10 (*PTEN*) is a tumor suppressor gene and one of the most important negative regulator of the *PI3K/AKT* signaling pathway [77][78]. *PTEN* is deleted in several types of cancers, such as prostate, endometrial, glioblastoma, breast, melanoma and colon [79][80][81]. Lung cancers are malignant tumors where *PTEN* deregulation plays a crucial role in tumor cell proliferation, metastasis process, and resistance to treatments. Beyond 40% of NSCLC, cases express loss of *PTEN* and it is related to poor prognosis, especially for *EGFR*-positive patients treated with EGFR-TKIs [82]. Various preclinical models have disclosed that *PTEN* inactivation could alter the pattern of response to EGFR-TKIs [34][83], namely Chevallier et al. reported a retrospective cohort trial of the influence of concurrent mutations on patients with advanced NSCLC treated with TKIs [34]. The authors found five patients harboring a resistance pathogen mutation in *PTEN*, who showed poor mPFS of 6.8 months. These finding are consistent with a recent report from Huang et al. [73]. Finally, VanderLaan et al. reported 5% (1/19 patients) of *PTEN/EGFR* altered patients [72].

It has been already proved that the downstream signaling pathway of the *HER* family phosphatidylinositol-3-kinase (PI3K) is related to carcinogenesis in lung cancer [11]. *PIK3CA* mutations are detected in almost 3–7% of patients with lung adenocarcinomas and commonly they are located in exons 9 and 20 [84]. These genetic aberrations generate constitutive activation of PI3K, AKT phosphorylation, and mTORC1 downstream which have a crucial role in cell survival and proliferation. In contrast to the mutual exclusivity of various oncogenic aberrations in NSCLCs, the coexistence of *PIK3CA* mutations with other oncogenic alterations is well established [84][85]. Actually, approximately 3.5% of *EGFR*-mutant patients harbor *PI3KCA* gene alterations and this seems to blunt the response to TKIs treatment. In vitro data suggest that EGFR-TKI sensitivity in *EGFR*-positive NSCLC cell lines has been related to downregulation of the PI3K pathway, and as a matter of fact increased resistance to gefitinib was confirmed after the introduction of the *PIK3CA* p.E545K mutation into a gefitinib-sensitive lung adenocarcinoma cell line [86]. Eng et al. analyzed the prognostic impact of a concurrent *PIK3CA* mutation in 13 *EGFR*-mutant NSCLC patients, finding poor ORR (62% vs. 83%; $p = 0.80$) and shorter median Time To Progression (TTP) (7.8 vs. 11.1 months; $p = 0.84$) to EGFR-TKIs [86]. Moreover, Wu et al. examined the significance and the effect of *PIK3CA* mutations on treatment outcomes to EGFR-TKIs of lung adenocarcinoma [41]. The study population included six *PIK3CA* mutation-positive patients. In contrast to the analysis by Eng et al., the authors reported similar response (ORR, 66.7 vs. 78.7%; $p = 0.476$) to EGFR-TKIs as wild-type patients. Notably, *PIK3CA*-mutant patients displayed a trend toward better PFS (12.0 vs. 8.8 months) and OS (25.1 vs. 21.4 months), still the variations were not statistically significant. Accordingly, Wang et al. investigated a cohort of 1117 NSCLC patients, out of which 17 patients harbored simultaneously a mutation in *EGFR* and *PIK3CA* [87]. They found that survival for patients with single *PIK3CA* mutation was poorer than patients harboring a concurrent double alteration in *PIK3CA* and *EGFR* ($p = 0.004$). Chevallier et al. reported two patients with double *EGFR/PIK3CA* alteration and poor survival [34]. De Marchi et al. detected 10/1208 individuals concurrent mutated in *EGFR* and *PIK3CA* [9], while Rachiglio et al. identified nine patients with double mutations displaying a mPFS of 5.5 months under EGFR-TKIs treatments [32]. Zhang et al. presented four patients harboring concurrent *EGFR/PIK3CA* genomic alteration [88],

whereas Li et al. reported 64 (3.3%) of their 5125 patients [10]. Additionally, Hu et al. described nine out of 320 patients and of note they reported the longest PFS of 7.6 months, while Chen et al. found three out of 36 patients describing lower ORR (43.75% vs. 80.0%; $p = 0.024$) comparing to the population with a single *EGFR* alteration [11]. Lammers et al. reported three cases among their study population with poor response to erlotinib treatment [89], whereas Huang et al. recently reported better ORR of 72% among the 18 patients harboring double concurrent genomic alteration under icotinib and anlotinib treatment.

CDKN2A gene encodes p16, a tumor suppressor which promotes a cell cycle arrest in G1 phase by inhibiting Rb phosphorylation. In NSCLC patients, the inactivation of *CDKN2A* is one of the most common genomic alterations detected [80], especially through the mechanisms of homozygous deletions (HDs), presented in up to 29–59% of lung adenocarcinomas regardless of the concurrent *EGFR* mutation [81]. Jiang et al. studied 127 *EGFR*-positive patients with NSCLC, identifying 31 out of 127 (24.4%) patients with HDs in *CDKN2A*, who displayed poor ORR to EGFR-TKIs and shorter mPFS. Of note, these results might justify the use of the combo EGFR-TKI and CDK4/6 inhibitors in this particular subset of patients [83]. Moreover, Chang et al. analyzed 31 NSCLC patients with *EGFR* alteration revealing copy number variation (CNV) loss *in CDKN2A* gene in seven patients (22.6%) [27]. Notably, four out of seven patients had an intermediate response (six to 12 months of PFS), while the other three patients presented a poor response (<six months). Finally, Skoulidis et al. and colleagues showed 24.6% of *CDKN2A* alterations in their cohort, concluding that co-alterations in *EGFR* and *CDKN2A* were related to EGFR TKIs acquired resistance [90].

RB1 gene is a regulator of cell cycle and is phosphorylated by CDK4/6 to S-phase entry [91]. The alterations in RB1 pathway have been associated to worse prognosis in NSCLC patients [90]. In their article, Sato et al. and colleagues investigated 43 patients with *EGFR* mutations revealing 16% (7/43) of *RB1* co-alterations [8]. Of note, these patients showed a poor prognosis. Hou et al. examined 71 NSCLC patients with *EGFR* mutations, of whom seven patients (9.9%) with a concomitant *RB1* alteration [92]. Moreover, it is well-established that *RB1* loss is a primary event correlated with transformation to Small-Cell Lung Cancer (SCLC) and consequently EGFR-TKIs treatment resistance [93][94]. Additionally, Yu et al. and Kim et al. reported *RB1* as one of the most common gene co-altered in NSCLC patients [63]. Particularly, Kim et al. and colleagues identified co-alteration in *RB1* as predictor of fast progression to TKI treatment [95].

4. Methods of Detection

The mutational analysis should be performed on tissue specimens and the most common methods for *EGFR* mutation detection with concomitant genomic alterations are reported in Table 1. Generally, the biological material available does not provide an amount of neoplastic cell percentage allowing the use of a Sanger Sequencing method. Conversely, high-sensitivity platforms as digital droplet PCR (ddPCR) (0.1%)[96] , or Amplification Refractory Mutation System (ARMS) with a specificity up to 1%[94]should be able to cleverly detect these pathogenetic variants with a specificity running up to wild-type DNA[94] . Nevertheless, the recent development of NGS accomplishes massive parallel gene mutation analysis and requires low amount of tissue, favoring the identification of several targetable molecular alterations until of 5% of VAF[96] .

Table 1. Summary of reported demographic characteristics of *EGFR*-positive NSCLC patients with concomitant genomic alterations.

Study	Study Type	Race	No. of Pts	Concurrent Genomic Alteration	Detection Method	Sample	VAF
Belardinilli et al. [2]	Case Report	Caucasian	1	*EGFR* complex	NGS	tumor tissue	40.30% 41.30% 67.50%
Benesova et al. [7]	Case Series	Caucasian	4	*EGFR+KRAS* *EGFR* complex	Sanger	tumor tissue	N/A
Fan et al. [22]	Case Report	Asian	1	*EGFR+ALK*	NGS	tumor tissue	EGFR 15.58% ALK 6.42%
Lammers et al. [89]	Case Report	Caucasian	1	*EGFR+PIK3CA*	SNapShot PCR	tumor tissue	N/A
Lee et al. [23]	Case Series	Asian	12	*EGFR+KRAS* *EGFR+ALK*	Sanger; Real Time PCR after PNA; FISH and IHC	tumor tissue	N/A
Miyanaga et al. [24]	Case Report	Asian	1	*EGFR+ALK*	PNA-LNA PCR clamp method, FISH and IHC	tumor tissue	N/A
Sweis et al. [25]	Case Series	Caucasian	4	*EGFR+ALK*	N/A	N/A	N/A
Thumallapally et al. [26]	Case Report	Caucasian	1	*EGFR+ALK*	FISH, direct sequencing	tumor tissue	N/A
Zhu et al. [35]	Case Report	Asian	1	*EGFR+ROS-1*	NGS, PCR and FISH	tumor tissue	N/A
Yang et al. [21]	Case Series	Asian	13	*EGFR+ALK*	IHC, FISH, Sanger, RT-PCR and RACE-PCR sequencing	tumor tissue	N/A
Hou et al. [71]	Retrospective	Asian	59	*EGFR+TP53* *EGFR+RB1*	NGS	tumor tissue	N/A

Study	Study Type	Race	No. of Pts	Concurrent Genomic Alteration	Detection Method	Sample	VAF
Zhu et al. [28]	Retrospective	Asian	2	*EGFR+ALK*	FISH, RT-PCR	tumor tissue	N/A
Li et al. [10]	Retrospective	Asian	149	*EGFR+ PIK3CA* *EGFR* complex *EGFR+KRAS* *EGFR+BRAF*	SurPlex®-xTAG70plex-EGFR liquidchip	tumor tissue	N/A
Liang et al. [6]	Retrospective	Asian	403	*EGFR* complex	NGS	tumor tissue + plasma	N/A
Liu et al. [97]	Retrospective	Asian	21	*EGFR+ALK*	NGS	tumor tissue + plasma	N/A
Nardo et al. [33]	Retrospective	Caucasian	3	*EGFR+KRAS*	ddPCR	tumor tissue + plasma	KRAS <0.2
Rachiglio et al. [32]	Retrospective	Caucasian	38	*EGFR+KRAS* *EGFR+BRAF* *EGFR+MET* *EGFR+TP53* *EGFR+PIK3CA*	NGS, ddPCR	tumor tissue + plasma	KRAS 2–38% EGFR ≥ 2%
Sato et al. [8]	Retrospective	Asian	43	*EGFR* complex *EGFR+TP53* *EGFR+RB1*	NGS	tumor tissue	N/A
VanderLaan et al. [72]	Retrospective	Caucasian	19	*EGFR+TP53* *EGFR+PIK3CA* *EGFR+PTEN*	NGS, Sanger	tumor tissue	N/A
Wu et al. [98]	Retrospective	Asian	12	*EGFR+PIK3CA*	Sanger, RT-PCR	tumor tissue	N/A
Zheng et al. [76]	Retrospective	Asian	11	*EGFR+TP53*	NGS	tumor tissue	N/A
Zhuang et al. [20]	Retrospective	Asian	43	*EGFR+ALK* *EGFR+ROS-1* *EGFR+KRAS* *EGFR+BRAF*	ARMS	tumor tissue	N/A

Study	Study Type	Race	No. of Pts	Concurrent Genomic Alteration	Detection Method	Sample	VAF
Huang et al. [73]	Prospective	Asian	18	*EGFR+TP53/PTEN* *EGFR+PIK3CA*	N/A	N/A	N/A
Zhang et al. [74]	Prospective	Asian	N/A	*EGFR+TP53*	NGS	N/A	N/A
Canale et al. [69]	Retrospective	Caucasian	136	*EGFR+TP53*	Sanger, MassARRAY, NGS	tumor tissue	N/A
Chang et al. [27]	Retrospective	Asian	26	*EGFR+ALK* *EGFR+TP53* *EGFR+PIK3CA* *EGFR+CDKN2A*	NGS, CNV	tumor tissue	N/A
Chen et al. [11]	Retrospective	Asian	16	*EGFR* complex *EGFR+ALK* *EGFR+KRAS* *EGFR+PIK3CA* *EGFR+TP53*	NGS	tumor tissue + plasma	N/A
De Marchi et al. [9]	Retrospective	Caucasian	47	*EGFR* complex *EGFR+KRAS* *EGFR+PIK3CA*	NGS, Sanger, SNP array	tumor tissue	N/A
Eng et al. [86]	Retrospective	Caucasian	13	*EGFR+PIK3CA*	mutation hotspot testing, FISH, multiplex sizing assays	tumor tissue	N/A
Chevallier et al. [34]	Retrospective	Caucasian	20	*EGFR+TP53* *EGFR+MET* *EGFR+KRAS* *EGFR+PIK3CA* *EGFR+PTEN*	NGS	tumor tissue	N/A
Hu et al. [19]	Retrospective	Asian	21	*EGFR+ALK* *EGFR+PIK3CA* *EGFR+KRAS* *EGFR+ROS-1* *EGFR+RET* *EGFR+HER2*	ARMS; adx-RT, mutation detection kit; fusion gene detection kit	tumor tissue	N/A
Chen et al. [12]	Retrospective	Asian	71	*EGFR* complex *EGFR+TP53* *EGFR+ALK*	NGS, ARMS	tumor tissue	N/A

Study	Study Type	Race	No. of Pts	Concurrent Genomic Alteration	Detection Method	Sample	VAF
				EGFR+BRAF *EGFR+MET*		+ plasma	
Lee et al. [5]	Retrospective	Asian	7	*EGFR+ALK* *EGFR+MET* *EGFR+TP53* *EGFR* complex	FISH, NGS, Sanger	tumor tissue	N/A
Zhang et al. [88]	Retrospective	Asian	9	*EGFR* complex *EGFR+KRAS* *EGFR+PIK3CA*	FISH, liquid chip platform	tumor tissue	N/A
Wang et al. [87]	Retrospective	Asian	17	*EGFR+PIK3CA*	Sanger, FISH, IHC	tumor tissue	N/A
Klempner et al. [55]	Case report	Asian	2	*EGFR+RET*	NGS	tumor tissue	53% 54% 62% 18%

Abbreviations: No, number; Pts, patients; VAF, variant allele frequency; NGS, next generation sequencing; N/A, not applicable; FISH, fluorescent in situ hybridization; IHC, immunohistochemistry; PCR, polymerase chain reaction; ARMS, amplification refractory mutation system; CNV, copy number variation; SNP, single nucleotide polymorphism; RT-PCR, real time-PCR; ddPCR, digital droplet PCR; RACE-PCR, rapid amplification cDNA ends PCR; PNA-LNA PCR, peptide nucleic acid-locked nucleic acid PCR.

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