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Review

Non-Small Cell Lung Cancer Harboring Concurrent *EGFR* Genomic Alterations: A Systematic Review and Critical Appraisal of the Double Dilemma

Valerio Gristina [1,†], Maria La Mantia [1,†], Antonio Galvano [1,†], Sofia Cutaia [1], Nadia Barraco [1], Marta Castiglia [1], Alessandro Perez [1], Marco Bono [1], Federica Iacono [1], Martina Greco [1], Katia Calcara [1], Valentina Calò [1], Sergio Rizzo [1], Lorena Incorvaia [1], Maria Chiara Lisanti [1], Giulia Santanelli [1], Delia Sardo [1], Sara Inguglia [1], Lavinia Insalaco [1], Luisa Castellana [1], Stefania Cusenza [1], Gianni Pantuso [1], Antonio Russo [1,*] and Viviana Bazan [2]

[1] Department of Surgical, Oncological and Oral Sciences, University of Palermo, 90127 Palermo, Italy; valerio.gristina@you.unipa.it (V.G.); maria.lamantia@you.unipa.it (M.L.M.); antonio.galvano@unipa.it (A.G.); sofia.cutaia@gmail.com (S.C.); barraconadia@gmail.com (N.B.); martacastiglia@gmail.com (M.C.); ale-like@libero.it (A.P.); marcobono29@gmail.com (M.B.); fed.iacono@gmail.com (F.I.); martinagreco21@virgilio.it (M.G.); katiushacalcara@gmail.com (K.C.); valentinacalo74@gmail.com (V.C.); sergiorizzo77@gmail.com (S.R.); lorena.incorvaia@unipa.it (L.I.); lisanti.chiara@gmail.com (M.C.L.); giuliasantanelli92@libero.it (G.S.); delia.sardo@gmail.com (D.S.); sara_inguglia@libero.it (S.I.); lavinia.insalaco@gmail.com (L.I.); luisa.castellana22@gmail.com (L.C.); stefaniacusenza1@gmail.com (S.C.); gianni.pantuso@unipa.it (G.P.)

[2] Department of Biomedicine, Neuroscience and Advanced Diagnostics (Bi.N.D.), Section of Medical Oncology, University of Palermo, 90127 Palermo, Italy; viviana.bazan@usa.net

* Correspondence: antonio.russo@usa.net

† These authors contributed equally to this work.

Abstract: The molecular pathways which promote lung cancer cell features have been broadly explored, leading to significant improvement in prognostic and diagnostic strategies. Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) have dramatically altered the treatment approach for patients with metastatic non-small cell lung cancer (NSCLC). Latest investigations by using next-generation sequencing (NGS) have shown that other oncogenic driver mutations, believed mutually exclusive for decades, could coexist in *EGFR*-mutated NSCLC patients. However, the exact clinical and pathological role of concomitant genomic aberrations needs to be investigated. In this systematic review, we aimed to summarize the recent data on the oncogenic role of concurrent genomic alterations, by specifically evaluating the characteristics, the pathological significance, and their potential impact on the treatment approach.

Keywords: NSCLC; NGS; *EGFR*; concurrent genomic alterations; systematic review

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1. Introduction

Lung cancer is the most predominant cancer type and is one of the driving causes of cancer-related death worldwide [1]. Non-small cell lung cancer (NSCLC) accounts for roughly 85–90% of overall cases of lung malignancies and includes different histological subtypes [2–4]. Recently, 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) [5–7]. *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 [8,9]. 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 [10,11]. 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. In fact, NGS allows to examine both DNA- and RNA-based aberrations, thus concurrently analyzing significant gene pathogenic variants [12–14]. Additionally, despite oncogenic driver alterations were considered to be mutually exclusive, current findings have called higher attention to the presence of coexisting genomic alterations in *EGFR*-positive NSCLC patients [15]. The clinical and pathological significance of co-existing driver genomic variants has not been yet elucidated, raising several questions on therapeutic options for these particular subsets of patients. In the current systematic review, we aimed to highlight the updated data on the oncogenic role of concurrent genomic alterations, by specifically evaluating the clinical characteristics, the pathological significance and their potential impact on the treatment approach.

2. Materials and Methods

The systematic review was performed conforming to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines (PRISMA) (Supplementary Figure S1) [16]. In March 2021, a MEDLINE and Cochrane database systematic literature search was conducted using the following search words "*EGFR*" OR "*ErbB 1* Receptor" AND "concurrent" OR "concomitant" OR "coexisting" AND ("lung" OR "Non-Small-Cell Lung"). We used the terms "*EGFR*" AND "concurrent" for the significant abstracts published on the American Society of Clinical Oncology (ASCO) and the European Society of Medical Oncology (ESMO) and ClinicalTrials.gov databases. The literature search involved the cited bibliography of the reviewed articles too. The entire search strategy can be found in the supplementary material (Supplementary Figure S2). We searched for clinical trials evaluating patients with histological diagnosis of unresectable or advanced *EGFR*-positive NSCLC and concurrent genetic alterations, including non-randomized, cohort, cross-sectional, retrospective and case-control studies. Furthermore, we also excluded other reviews (systematic or not) and meta-analyses. Moreover, non-peer-reviewed publications, like abstracts displayed in conferences and meetings were taken into consideration. We excluded research trials conducted on animals, preclinical trials, as well as phase 1 and 2 trials. We reported demographics and clinical information about the included studies, such as concurrent genomic alteration, race of the study population, detection method, sample, variant allele frequency (VAF), treatments, clinical outcomes. The language of the data collected was limited to English. All data collected with the above-mentioned search strategy were reviewed by two authors (M.L.M. and V.G.), who independently screened and selected abstracts and titles according to the aforementioned exclusion and inclusion criteria. Disagreements were discussed and finally solved with a third author (A.G.).

3. Results

The systematic literature search identified a total of 827 records. The literature data collected through the systematic databases search underwent two exclusion steps: The first being based on title and abstract, whereas the second being subject to an exhaustive read-through. Additionally, in the case that an article did not conform to the inclusion and exclusion criteria, it was discarded. Thus, 11 records were excluded because of duplicates, while 56 records were finally ruled out being reviews, letters, commentaries, editorials, or protocols. Moreover, five full text papers were not available in English, thence excluded. After this process, 634 data met the eligibility criteria, whereas 600 were excluded since no data of interest were reported (Figure 1). Finally, a total of 36 studies met our inclusion and exclusion criteria; thus, they were included in the systematic research of the literature (see Table 1). Namely, a total of 11 case reports, two abstracts and 23 original research articles considered have examined concurrent *EGFR* mutations and their potential impact on NSCLC patients. Particularly, a total of 1313 patients harbored a double concomitant EGFR genomic alteration. Principally, the co-existing mutations identified are on-target

EGFR gene alterations, *TP53*, *PIK3CA*, *PTEN*, *RB1* and *CDKN2A*; whereas concomitant actionable driver aberrations, within anaplastic lymphoma kinase (*ALK*), c-ros oncogene-1 (*ROS-1*), v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*), mesenchymal epithelial transition (*MET*), Rearranged during transfection (*RET*) and Kirsten rat sarcoma viral oncogene homolog (*KRAS*) genes, are less comprehensively represented (Figure 2). Indeed, regarding complex *EGFR* mutation, we found two case reports and eight original articles reporting complex *EGFR* mutation in the study population. Concurrent *TP53* and *EGFR* mutations were described in 12 articles and two abstracts, and *PI3KCA/EGFR* co-alterations were reported in 11 articles, one abstract and a single case report, whereas only three research articles and one abstract included patient harboring co-existing *PTEN/EGFR* aberrations, two papers presented original data on *CDKN2A/EGFR* concomitant alterations, and two articles evaluated *RB1/EGFR* co-existing genomic alterations. Moreover, the systematic review of the literature identified only a single article including a concurrent *BRAF/EGFR* mutant patient, two works reporting *MET* concurrent *EGFR* alterations, a single case report and an original research article evaluating *EGFR/RET* concurrent alterations, and three papers evaluating *ROS-1/EGFR* concomitant alterations. Finally, six original research articles and five case reports were conducted on concurrent oncogenic driver *ALK* and *EGFR* aberrations, while two reports and five original articles evaluated concomitant *EGFR/KRAS* mutations. Tables 1 and 2 summarize the demographic characteristics and reported treatment outcomes of patients with NSCLC and double genetic alterations, respectively.

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. [17]	Case Report	Caucasian	1	*EGFR* complex	NGS	tumor tissue	40.30% 41.30% 67.50%
Benesova et al. [18]	Case Series	Caucasian	4	*EGFR+KRAS* *EGFR* complex	Sanger	tumor tissue	N/A
Fan et al. [19]	Case Report	Asian	1	*EGFR+ALK*	NGS	tumor tissue	EGFR 15.58% ALK 6.42%
Lammers et al. [20]	Case Report	Caucasian	1	*EGFR+PIK3CA*	SNapShot PCR	tumor tissue	N/A
Lee et al. [21]	Case Series	Asian	12	*EGFR+KRAS* *EGFR+ALK*	Sanger; Real Time PCR after PNA; FISH and IHC	tumor tissue	N/A
Miyanaga et al. [22]	Case Report	Asian	1	*EGFR+ALK*	PNA-LNA PCR clamp method, FISH and IHC	tumor tissue	N/A
Sweis et al. [23]	Case Series	Caucasian	4	*EGFR+ALK*	N/A	N/A	N/A
Thumallapally et al. [24]	Case Report	Caucasian	1	*EGFR+ALK*	FISH, direct sequencing	tumor tissue	N/A
Zhu et al. [25]	Case Report	Asian	1	*EGFR+ROS-1*	NGS, PCR and FISH	tumor tissue	N/A
Yang et al. [26]	Case Series	Asian	13	*EGFR+ALK*	IHC, FISH, Sanger, RT-PCR and RACE-PCR sequencing	tumor tissue	N/A
Hou et al. [27]	Retrospective	Asian	59	*EGFR+TP53* *EGFR+RB1*	NGS	tumor tissue	N/A
Zhu et al. [28]	Retrospective	Asian	2	*EGFR+ALK*	FISH, RT-PCR	tumor tissue	N/A
Li et al. [29]	Retrospective	Asian	149	*EGFR+ PIK3CA* *EGFR* complex *EGFR+KRAS* *EGFR+BRAF*	SurPlex®-xTAG70plex-EGFR liquidchip	tumor tissue	N/A

Table 1. *Cont.*

Study	Study Type	Race	No. of Pts	Concurrent Genomic Alteration	Detection Method	Sample	VAF
Liang et al. [30]	Retrospective	Asian	403	*EGFR* complex	NGS	tumor tissue + plasma	N/A
Liu et al. [31]	Retrospective	Asian	21	*EGFR+ALK*	NGS	tumor tissue + plasma	N/A
Nardo et al. [32]	Retrospective	Caucasian	3	*EGFR+KRAS*	ddPCR	tumor tissue + plasma	KRAS <0.2
Rachiglio et al. [33]	Retrospective	Caucasian	38	*EGFR+KRAS* *EGFR+BRAF* *EGFR+MET* *EGFR+TP53* *EGFR+PIK3CA*	NGS, ddPCR	tumor tissue + plasma	KRAS 2–38% EGFR $\geq$ 2%
Sato et al. [34]	Retrospective	Asian	43	*EGFR* complex *EGFR+TP53* *EGFR+RB1*	NGS	tumor tissue	N/A
VanderLaan et al. [35]	Retrospective	Caucasian	19	*EGFR+TP53* *EGFR+PIK3CA* *EGFR+PTEN*	NGS, Sanger	tumor tissue	N/A
Wu et al. [36]	Retrospective	Asian	12	*EGFR+PIK3CA*	Sanger, RT-PCR	tumor tissue	N/A
Zheng et al. [37]	Retrospective	Asian	11	*EGFR+TP53*	NGS	tumor tissue	N/A
Zhuang et al. [38]	Retrospective	Asian	43	*EGFR+ALK* *EGFR+ROS-1* *EGFR+KRAS* *EGFR+BRAF*	ARMS	tumor tissue	N/A
Huang et al. [39]	Prospective	Asian	18	*EGFR+TP53/PTEN* *EGFR+PIK3CA*	N/A	N/A	N/A
Zhang et al. [40]	Prospective	Asian	N/A	*EGFR+TP53*	NGS	N/A	N/A
Canale et al. [41]	Retrospective	Caucasian	136	*EGFR+TP53*	Sanger, MassARRAY, NGS	tumor tissue	N/A
Chang et al. [42]	Retrospective	Asian	26	*EGFR+ALK* *EGFR+TP53* *EGFR+PIK3CA* *EGFR+CDKN2A*	NGS, CNV	tumor tissue	N/A
Chen et al. [43]	Retrospective	Asian	16	*EGFR* complex *EGFR+ALK* *EGFR+KRAS* *EGFR+PIK3CA* *EGFR+TP53*	NGS	tumor tissue + plasma	N/A
De Marchi et al. [44]	Retrospective	Caucasian	47	*EGFR* complex *EGFR+KRAS* *EGFR+PIK3CA*	NGS, Sanger, SNP array	tumor tissue	N/A
Eng et al. [45]	Retrospective	Caucasian	13	*EGFR+PIK3CA*	mutation hotspot testing, FISH, multiplex sizing assays	tumor tissue	N/A
Chevallier et al. [46]	Retrospective	Caucasian	20	*EGFR+TP53* *EGFR+MET* *EGFR+KRAS* *EGFR+PIK3CA* *EGFR+PTEN*	NGS	tumor tissue	N/A
Hu et al. [47]	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

Table 1. *Cont.*

Study	Study Type	Race	No. of Pts	Concurrent Genomic Alteration	Detection Method	Sample	VAF
Chen et al. [48]	Retrospective	Asian	71	*EGFR* complex *EGFR+TP53* *EGFR+ALK* *EGFR+BRAF* *EGFR+MET*	NGS, ARMS	tumor tissue + plasma	N/A
Lee et al. [49]	Retrospective	Asian	7	*EGFR+ALK* *EGFR+MET* *EGFR+TP53* *EGFR* complex	FISH, NGS, Sanger	tumor tissue	N/A
Zhang et al. [50]	Retrospective	Asian	9	*EGFR* complex *EGFR+KRAS* *EGFR+PIK3CA*	FISH, liquid chip platform	tumor tissue	N/A
Wang et al. [51]	Retrospective	Asian	17	*EGFR+PIK3CA*	Sanger, FISH, IHC	tumor tissue	N/A
Klempner et al. [52]	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.

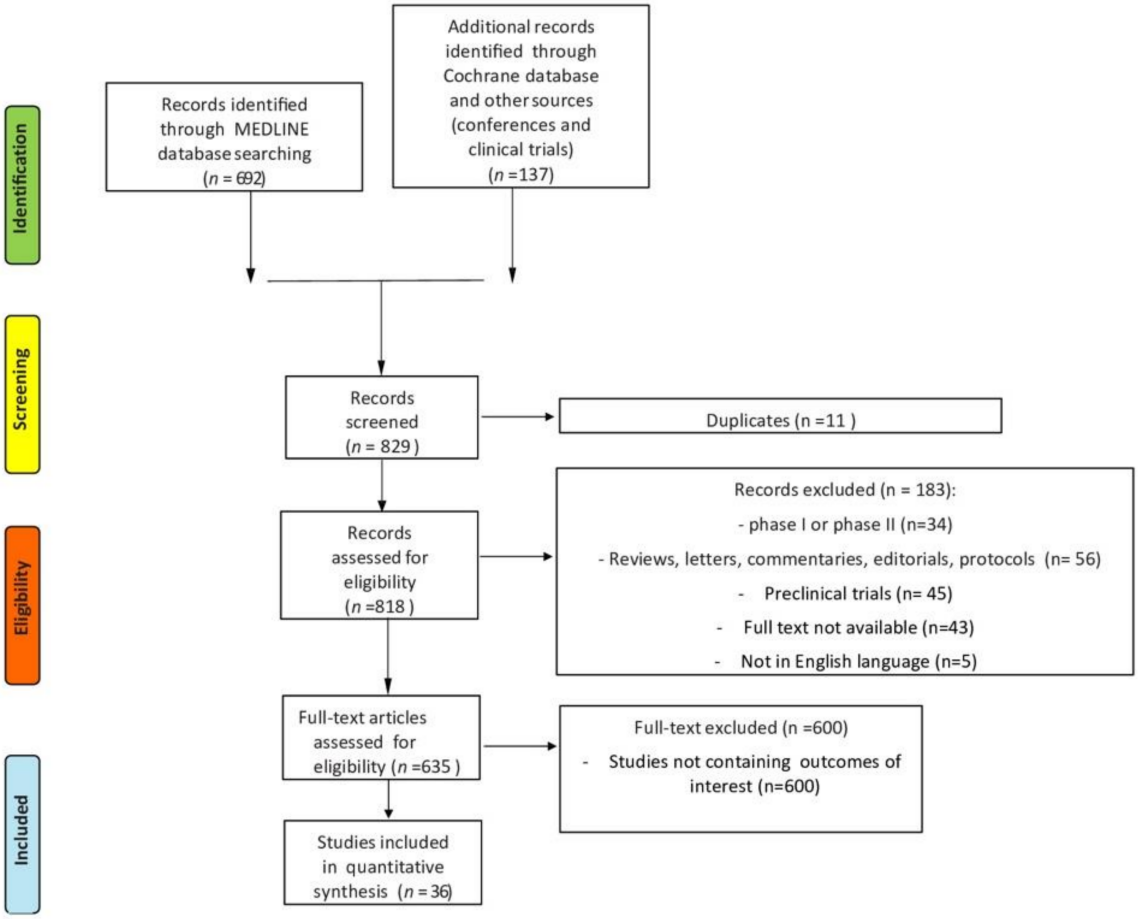

Figure 1. Preferred reporting items for systematic reviews and meta-analyses (PRISMA) flowchart diagram.

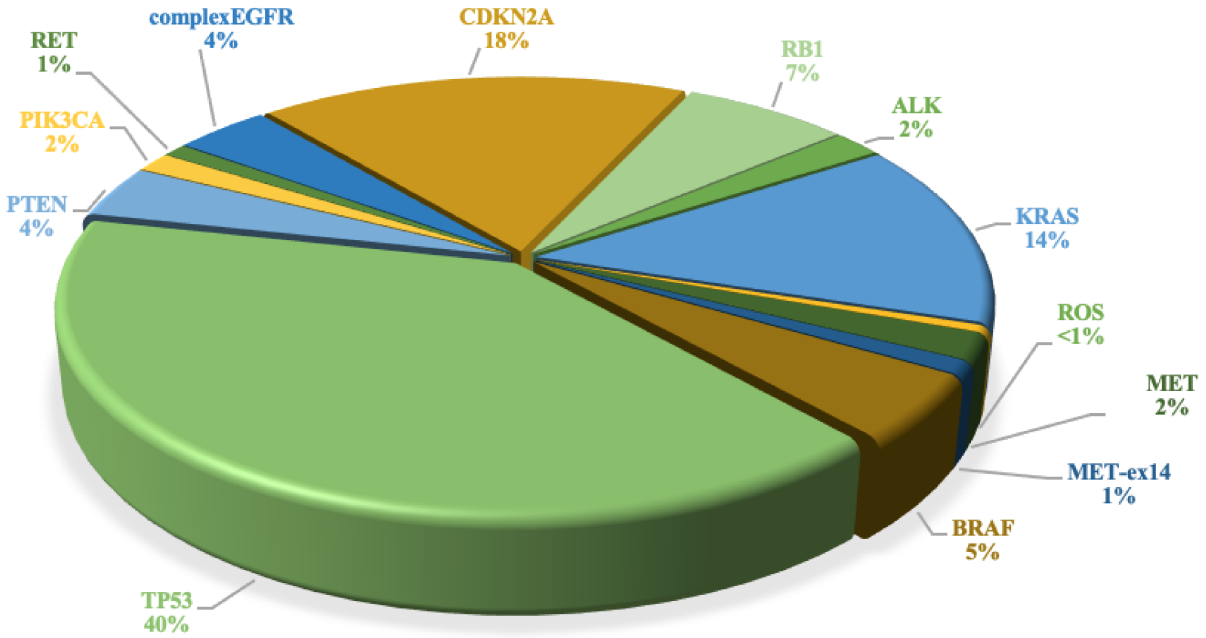

Figure 2. Distribution of the different concurrent *EGFR* mutations pathways.

Table 2. Summary of reported treatment outcomes of NSCLC patients with double concurrent genetic alterations.

Study	Concurrent Genomic Alteration	TKI	mPFS (mo.)	mOS (mo.)	Best Response
Belardinilli et al. [17]	*EGFR* complex	Afatinib	8	N/A	PR
Benesova et al. [18]	*EGFR+KRAS* *EGFR* complex	Gefitinib Erlotinib	6–12	5-23	3 PR 1 CR
Fan et al. [19]	*EGFR+ALK*	Gefitinib Crizotinib	18	N/A	PR/SD
Lammers et al. [20]	*EGFR+PIK3CA*	Erlotinib Afatinib PI3K inhibitor	1–4	N/A	SD/PR
Lee et al. [21]	*EGFR+KRAS* *EGFR+ALK*	Gefitinib Erlotinib Crizotinib	4–29	N/A	SD/PR
Miyanaga et al. [22]	*EGFR+ALK*	Gefitinib Erlotinib Crizotinib	2–7	N/A	SD
Sweis et al. [23]	*EGFR+ALK*	Erlotinib Crizotinib	2–12	N/A	PR/PD
Thumallapally et al. [24]	*EGFR+ALK*	Crizotinib	N/A	3 wk	N/A
Zhu et al. [25]	*EGFR+ROS-1*	Adj CT	N/A	N/A	N/A
Yang et al. [26]	*EGFR+ALK*	Gefitinib Erlotinib Crizotinib Afatinib	12–27.4	N/A	SD/PR/PD
Hou et al. [27]	*EGFR+TP53* *EGFR+RB1*	Erlotinib Gefitinib Icotinib	4–11	10–59	N/A
Zhu et al. [28]	*EGFR+ALK*	N/A	N/A	N/A	N/A

Table 2. *Cont.*

Study	Concurrent Genomic Alteration	TKI	mPFS (mo.)	mOS (mo.)	Best Response
Li et al. [29]	*EGFR+PIK3CA* *EGFR* complex *EGFR+KRAS* *EGFR+BRAF*	N/A	N/A	N/A	N/A
Liang et al. [30]	*EGFR Complex*	N/A	N/A	N/A	N/A
Liu et al. [31]	*EGFR+ALK*	Osimertinib Crizotinib Afatinib	6–15	N/A	N/A
Nardo et al. [32]	*EGFR+KRAS*	Erlotinib Gefitinib Afatinib	5	6	PR
Rachiglio et al. [33]	*EGFR+KRAS* *EGFR+BRAF* *EGFR+MET* *EGFR+TP53* *EGFR+PIK3CA*	Erlotinib Gefitinib Afatinib	7	15.5	N/A
Sato et al. [34]	*EGFR* complex *EGFR+TP53* *EGFR+RB1*	Gefitinib Erlotinib	N/A	N/A	PR
VanderLaan et al. [35]	*EGFR+TP53* *EGFR+PIK3CA* *EGFR+PTEN*	Erlotinib Gefitinib Afatinib	6.5	15.5	N/A
Wu et al. [36]	*EGFR+PIK3CA*	Erlotinib Gefitinib Afatinib	12	5.1	PR
Zheng et al. [37]	*EGFR+TP53*	N/A	N/A	23.9	N/A
Zhuang et al. [38]	*EGFR+ALK* *EGFR+ROS-1* *EGFR+KRAS* *EGFR+BRAF*	Gefitinib Erlotinib Afatinib Icotinib Crizotinib Alectinib	9.6	N/A	PR
Huang et al. [39]	*EGFR+TP53/PTEN* *EGFR+PIK3CA*	Anlotinib Icotinib	N/A	N/A	PR
Zhang et al. [40]	*EGFR+TP53*	Gefitinib Afatinib	N/A	N/A	N/A
Canale et al. [41]	*EGFR+TP53*	Erlotinib Gefitinib Afatinib	5.8–12.9	29.7–19.5	CR
Chang et al. [42]	*EGFR+ALK* *EGFR+TP53* *EGFR+PIK3CA* *EGFR+CDKN2A*	Erlotinib Gefitinib Afatinib	1–24.2	5.4–57.6	N/A
Chen et al. [43]	*EGFR* complex *EGFR+ALK* *EGFR+KRAS* *EGFR+PIK3CA* *EGFR+TP53*	Erlotinib Gefitinib Afatinib Osimertinib	18.7	N/A	CR
De Marchi et al. [44]	*EGFR* complex *EGFR+KRAS* *EGFR+PIK3CA*	N/A	N/A	N/A	N/A

Table 2. *Cont.*

Study	Concurrent Genomic Alteration	TKI	mPFS (mo.)	mOS (mo.)	Best Response
Eng et al. [45]	*EGFR+PIK3CA*	Gefitinib	7.8	18	PR
Chevallier et al. [46]	*EGFR+TP53* *EGFR+MET* *EGFR+KRAS* *EGFR+PIK3CA* *EGFR+PTEN*	Erlotinib Gefitinib Afatinib Osimertinib	6.8–11.6	7.7–16.8	N/A
Hu et al. [47]	*EGFR+ALK* *EGFR+PIK3CA* *EGFR+KRAS* *EGFR+ROS-1* *EGFR+RET* *EGFR+HER2*	Erlotinib Gefitinib Crizotinib Icotinib	1–24	10–43	PR/PD
Chen et al. [48]	*EGFR* complex *EGFR+TP53* *EGFR+ALK* *EGFR+BRAF* *EGFR+MET*	Erlotinib Gefitinib Icotinib	6–24	N/A	N/A
Lee et al. [49]	*EGFR+ALK* *EGFR+MET* *EGFR+TP53* *EGFR* complex	Erlotinib Gefitinib Crizotinib	1–2.1	1–21.8	N/A
Zhang et al. [50]	*EGFR* complex *EGFR+KRAS* *EGFR+PIK3CA*	N/A	N/A	N/A	N/A
Wang et al. [51]	*EGFR+PIK3CA*	Gefitinib	N/A	N/A	PR
Klempner et al. [52]	*EGFR+RET*	Erlotinib	N/A	N/A	PR/PD

Abbreviations: TKI, tyrosine kinase inhibitor; mPFS, median progression-free survival; mOS, median overall survival; PR, partial response; CR, complete response; SD, stable disease; PD, progressive disease; N/A, not applicable; Adj CT, adjuvant chemotherapy; mo., months; wk, weeks.

3.1. 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 [8,17]. 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 [53]. Complex *EGFR* mutations occur almost in 3–7% of *EGFR*-mutant patients [54]. 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 [17]. 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 [49]. 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 [30]. 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 [54]. 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 [18]. 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 [34]. 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 [44]. Li et al. detected 58/5125 *EGFR* double mutations, with the highest incidence rate of p.T790M and p.L858R [29]. Chen et al. presented 4/36 patients harboring concurrent 19del and p.L858R with a worse response after TKI treatment [43]. Additionally, Chen et al. reported concurrent *EGFR* complex genomic alterations in 20 patients with the worst outcome in terms of OS [48].

3.2. 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.

3.2.1. *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) [55]. In 2007 *ALK* fusion was described in lung adenocarcinoma for the first time in a limited cohort of Asian individuals [56]. 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 [57]. Consequently, the fusion *EML4-ALK* with tyrosine kinase function stimulates proliferation and cell survival [57]. Chromosomal rearrangements in the *ALK* gene are detected in approximately 5% of NSCLC patients [58]. Moreover, this driver fusion is predominantly estimated mutually exclusive with other genetic mutations, such

as *EGFR* [59]. 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 [59,60]. Liu et al. evaluated the efficacy of TKI treatments on 21 co-altered *EGFR* and *ALK* patients with advanced NSCLC [60]. 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 [47]. 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 [38]. Yang et al. assessed that 13/977 NSCLC patients screened harbored a concomitant genetic aberration in *EGFR* and *ALK* genes [26]. 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 [19]. 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 [21]. Notably, Miyanaga et al. described a single case where the patient showed response both to first-generation EGFR-TKIs and crizotinib [22]. Sweis et al. presented a case series including four patients treated with erlotinib and crizotinib, achieving a stable disease as the best response [23]. 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 [24]. In their exploratory study, Lee et al. found two out of 197 *EGFR*-positive NSCLC patients with a concurrent genomic alteration in *ALK* [49]. 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 [42], 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 [48].

3.2.2. *KRAS*

KRAS alterations are frequently represented by missense mutations occurring in lung adenocarcinomas [61]. 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 [61]. Moreover, almost 6–35% of *EGFR* positive patients harbor a concomitant genetic aberration in the *KRAS* gene [62]. P.G12C, p.G12V, and p.G12D mutation are the most frequent alteration detected [63]. 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 [18]. 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 [38]. 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 [33]. 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 [32]. 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 [21], as Li et al. who reported 30 patients with double alterations out of a cohort of 5125 individuals [29]. Chevallier et al. described a single case [46], as De Marchi et al. [44]. Moreover, Zhang et al. found two out of 120 patients with double concurrent genomic aberrations [54]. Whereas Hu et al. described a single case of *EGFR/KRAS* out of a cohort including 320 individuals [47], of note the patient showed progression after treatment with erlotinib. Finally, in the trial by Chen et al. [48], seven out of 36 patients displayed a concurrent alteration in *EGFR* and *ALK* with poorer PFS after EGFR-TKI treatment.

3.2.3. *ROS-1*

ROS-1 rearrangements has been detected in almost 1–2% of lung adenocarcinoma [25]. The ALK-TKI crizotinib is highly active in *ROS1*-rearranged patients [64]. 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 [25]. 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) [38]. 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 [43].

3.2.4. *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 [65]. *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 [66]. *MET* amplification is a well-known resistance mechanism against EGFR-TKIs, including the third-generation osimertinib [67,68]. 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 [66]. 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 [69,70]. 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) [69]. Additionally, Oxnard et al. examined the safety of osimertinib in combination with selumetinib/savolitinib/durvalumab [68]. Indeed, only three patients harbored *MET* amplification and p.T790M and they were treated with selumetinib displaying partial

response [68]. 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 [70]. 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 [46]. Lee et al. described a single patient with *MET* amplification >15 gene copies in 17% of tumor cells [49]. Chen et al. reported a single case including in the short PFS group (10% vs. 33% $p = 0.018$) [48]. 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.

3.2.5. *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 [71]. 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 [72,73]. Concomitant *EGFR/BRAF* aberrations are found in approximately 11% of EGFR-positive NSCLC patients, with the *BRAF* p.V600E mutation most frequently identified [74,75]. Chen et al. retrospectively screened 423 NSCLC patients harboring *EGFR* 19Del or p.L858R mutations reporting only one patient with concurrent *BRAF* p.V600E [48]. 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 [29]. Moreover, Rachiglio et al. found hotspot mutation in several genes, including *BRAF* in 14 patients (21.8%) of their cohort [33]. Zhuang et al. described two cases of concomitant *EGFR/BRAF* alteration, showing better outcomes with EGFR-TKI than with standard chemotherapy [38].

3.2.6. *RET*

Rearranged during transfection (*RET*) gene rearrangements are detected in almost 1% of NSCLC patients [76,77]. 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 [78,79]. In up to 10% of NSCLC patients under osimertinib treatment, oncogenic fusions of *RET* gene have been considered responsible for acquired resistance [78,80]. 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) [81,82]. 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 [47]. 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 [52]. 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.

3.3. 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 [83–85]. Inactivating mutations of the *TP53* gene affect the normal transcriptional p53 activity leading to tumor susceptibility and hinder patients' response to chemotherapy treatments [86,87]. Moreover, *TP53* alterations might be related to a poor prognosis in NSCLC patients [88]. Almost 55–65% of *EGFR*-positive NSCLC patients harbor a *TP53* coexisting mutation [49,89,90]. Preclinical models have already demonstrated a correlation between *TP53* mutation and response to EGFR-TKIs therapy [90–92]; 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 [91,93–95]. 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 [41]. 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 [95,96]. 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. [27]. 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. [35] 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 [48]. 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 [94]. 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% [39]. 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) [40,97]. 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 [33]. Interestingly, Sato et al. reported 12 patients (28%) with *EGFR/TP53* alteration [34]. 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 [37]. Lee et al. described three cases out of 197 patients [49]. Chevallier et al. reported 15 cases of double mutation, with no difference of survival [46]. Chang et al. found that *TP53* was the most common concomitant alteration detected (10/31 patients) [42], as Chen et al. reported in their study [48].

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 [98,99]. *PTEN* is deleted in several types of cancers, such as prostate, endometrial, glioblastoma, breast, melanoma and colon [100–102]. 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 [103]. Various preclinical models have disclosed that *PTEN* inactivation could alter the pattern of response to EGFR-TKIs [46,104], namely Chevallier et al. reported a retrospective cohort trial of the influence of concurrent mutations on patients with advanced NSCLC treated with TKIs [46]. 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. [39]. Finally, VanderLaan et al. reported 5% (1/19 patients) of *PTEN/EGFR* altered patients [35].

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 [43]. *PIK3CA* mutations are detected in almost 3–7% of patients with lung adenocarcinomas and commonly they are located in exons 9 and 20 [105]. 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 [105,106]. 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 [45]. 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 [45]. Moreover, Wu et al. examined the significance and the effect of *PIK3CA* mutations on treatment outcomes to EGFR-TKIs of lung adenocarcinoma [69]. 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* [51]. 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 [46]. De Marchi et al. detected 10/1208 individuals concurrent mutated in *EGFR* and *PIK3CA* [44], while Rachiglio et al. identified nine patients with double mutations displaying a mPFS of 5.5 months under EGFR-TKIs treatments [33]. Zhang et al. presented four patients harboring concurrent *EGFR/PIK3CA* genomic alteration [50], whereas Li et al. reported 64 (3.3%) of their 5125 patients [29]. 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 [43]. Lammers et al. reported three cases among their study population with poor response to erlotinib treatment [20], 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 [101], especially through the mechanisms of homozygous deletions (HDs), presented in up to 29–59% of lung adenocarcinomas regardless of the concurrent *EGFR* mutation [102]. 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 [104]. 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%) [42]. 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 [107].

RB1 gene is a regulator of cell cycle and is phosphorylated by CDK4/6 to S-phase entry [108]. The alterations in RB1 pathway have been associated to worse prognosis in NSCLC patients [107]. In their article, Sato et al. and colleagues investigated 43 patients with *EGFR* mutations revealing 16% (7/43) of *RB1* co-alterations [34]. 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 [109]. 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 [110,111]. Additionally, Yu et al. and Kim et al. reported *RB1* as one of the most common gene co-altered in NSCLC patients [90]. Particularly, Kim et al. and colleagues identified co-alteration in *RB1* as predictor of fast progression to TKI treatment [112].

3.4. 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%) [113], or Amplification Refractory Mutation System (ARMS) with a specificity up to 1% [111] should be able to cleverly detect these pathogenetic variants with a specificity running up to wild-type DNA [111]. 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 [113].

4. Discussion

Over the last decades, our treatment approach to lung cancer patients has been dramatically changed as a result of the development and clinical implementation of an essential

tool as NGS. *EGFR* mutations represent a molecular target in this particular population. In this context, oncogenic driver mutations in NSCLC were historically considered mutually exclusive, thus the potential association of two or more oncogenic driver aberrations has been poorly explored. Moreover, the advent of comprehensive genomic profiling in clinical samples enabled the detection of a significant number of concurrent alterations in *EGFR*-mutated NSCLC. Consequently, we perform a systematic review of the literature on concurrent genomic alterations and their oncogenic role to provide a deeper insight into the molecular heterogeneity of *EGFR*-mutated NSCLCs. Finally, the results of our systematic review of the literature seemed to indicate that EGFR-mutant NSCLC is not a single oncogene driven entity. Most of the results of our systematic review consisted in articles and reports on Asian population. This is consistent with the major prevalence of EGFR genomic alteration in NSCLC Asian patients [114,115]. Notable, Asian patients appeared to display better response comparing to Caucasian cohorts (see Table 2); this finding shows racial differences in genetic pathways and prompts further studies on this field of research.

Notably, the presence of coexisting genetic alteration might likely justify resistance to TKIs treatment [116]. Particularly, different clinical trials assessed that the concurrent presence of mutation provides a worse prognosis in *EGFR*-positive NSCLC patients treated with first-, second-, and third-generation TKIs. Indeed, these recent findings highlighted that *EGFR*-mutated tumors have notable intratumor heterogeneity with concomitant evidence of significant oncogenic gene aberrations [54,117]. In fact, through the use of NGS, Belardinilli et al. detected similar VAF of the pair of mutations located in exon 21, likely indicating a co-occurrence within the same tumor cells [17]. Accordingly, it is intriguing to speculate that the above-mentioned particular oncogenetic pattern identified in this lung cancer patient could explain the increased response to TKI treatment with afatinib, generally not found in NSCLC patients harboring complex *EGFR* alterations. Moreover, this interesting analysis pointed out the urgent need of further investigations in order to clarify the mechanism of differential responses to TKIs. In the study by Zhang et al. [54], patients with a sensitive *EGFR* alteration such as 19Del/p.L858R/p.L861Q, plus a p.T790M de novo or an exon 20 insertion exhibited the worst clinical outcomes [116], [118]. This could be clarified as they were treated with first- and second-generation EGFR-TKIs. It would have been intriguing evaluating patient's response after a third-generation EGFR-TKI treatment, like osimertinib. Indeed, based on the results of the FLAURA trial, osimertinib is considered the current standard of care for *EGFR* p.T790M positive patients. However, NSCLC patients with *EGFR* exon 20 insertion designate still a crucial unmet need. Recent preliminary data presented on March 2021 at ESMO Targeted Anticancer Therapies (TAT) Virtual Congress by Sacher et al. demonstrated that poziotinib, initially conceived as a HER2-inhibitor, has significant clinical activity on this particular subset of patients [119]. Although further evaluations are warranted, one could speculate that *EGFR*-positive patients harboring a complex mutation, alike an exon 20 insertion and a sensitive genetic alteration (19Del/p.L858R/p.L861Q) might benefit from a treatment with a potent irreversible TKI, such as poziotinib. Collectively, it is challenging to estimate the efficacy of *EGFR*-TKIs in NSCLC patients harboring uncommon complex *EGFR* genetic alterations due to the great heterogeneity of the mutations detected [119,120]. Previous clinical trials have evaluated that first-generation *EGFR*-TKIs showed poor efficacy for uncommon mutations (alone or plus a compound mutation) [121]. Consequently, afatinib and osimertinib should be taken into consideration for treatment of these patients. In fact, based on the durable responses demonstrated in the multicenter randomized clinical trials LUX-Lung 2, LUX-Lung 3, and LUX-Lung 6, on January 2018, the Food and Drug Administration (FDA) granted approval to afatinib as a first-line treatment option for patients carrying an uncommon mutation [69,122–125]. Additionally, osimertinib confirmed favorable activity in patients with NSCLC harboring uncommon *EGFR* mutations, as reported by Cho et al. [126,127]. Therefore, a treatment with afatinib or osimertinib seems to be a better strategy for this particular subset of patients; however, because of the high molecular heterogeneity and

low prevalence of this mutational pattern further clinical trials with larger sample size are warranted. 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 [25,44,65]. Zhuang et al. found that ALK-TKI therapy was more active as a first-line treatment than in later lines [39], while Yang et al. detected that patients appeared to better respond to EGFR-TKIs as a first-line setting. The great heterogeneity of clinical outcomes might be correlated with different levels of EGFR or ALK protein phosphorylation. The responses to EGFR- and/or ALK-TKIs appeared to be conflicting, thus it is recommended to fully evaluate by using high-sensitivity molecular techniques and detect the VAF in order to reconstruct the clonal architecture and heterogeneity. Despite further investigations are warranted, a combination of EGFR- and ALK-TKI might be considered a reasoned treatment strategy. Moreover, several cases have been reported *EGFR* and *KRAS* concurrent alterations. Given the potential impact of multiclonal characters of NSCLC on treatments, VAF quantitative estimation of both genetic mutations appears to be the best method able to determine who would benefit most from EGFR-TKI treatment. Interestingly, based on our findings, one could speculate that the coexistence of *EGFR/ROS-1* alteration is consistent with tumor tissue heterogeneity. Overall, due to the limited data available further larger studies remain mandatory in order to assess the treatment outcomes of patients harboring an *EGFR/ROS1* concurrent alteration. The impact of *EGFR/MET* co-alterations in NSCLC patients still represents an area of active investigation, yet larger clinical trials using uniform criteria to evaluate MET status are required [66]. Methods universally standardized in order to detect *MET* gene alteration are fluorescence in situ hybridization (FISH), immunohistochemistry (IHC), NGS, and real-time PCR, however the latter technique is not selective for cancel cells [123]. Consequently, the conflicting results in *MET* positivity detection might be attributed to the lack of data harmonization platform as well as of systematic criteria. Two commonly used scoring systems for assessing *MET* amplification are the Cappuzzo scoring system and the PathVysion [127–130]. It is well-established that activation of the *MET* pathway is one of the main acquired resistance mechanisms in EGFR-mutant patients, hence it is conceivable that a combination of EGFR- and MET-TKIs might have some activity in this particular subset of patients [48]. Moreover, several clinical trials have reported a secondary *BRAF* p.V600E mutation as a potential resistance mechanism to osimertinib treatment in *EGFR*-mutant patients. Meng et al. reported two patients with a p.T790 M mutation both treated with osimertinib who acquired a *BRAF* p.V600E mutation at PD [74]. Interestingly, a combination of dabrafenib and trametinib plus osimertinib was administered. One patient showed PR with a PFS of 14 months, whereas the second patient discontinued treatment due to severe pneumonitis. However, combined treatment with dabrafenib, trametinib and osimertinib appeared to be effective. Since literature data on the activity of these combined approaches are limited, further investigation represents an important issue. Furthermore, the worse prognosis of concurrent *EGFR/TP53* positive patients could be explained as genomic complex tumors might trigger different pathways bypassing *EGFR* as a target. Furthermore, among overall clinical studies, EGFR-TKIs appeared to have less activity in 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, as suggested by several trials [39,40,99]. 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 [131]. Collectively, the concurrence of genetic aberrations in different genes might be responsible for the sub clonal heterogeneity, thence it could justify the primary resistance to EGFR-TKI treatments in this particular subset of patients. Based on our findings, the impact of *PIK3CA* mutations on survival in *EGFR*-mutant patients is still under debate. On March 2021, Lage et al. presented a retrospective analysis of 1745 NSCLC patients receiving treatment from 2011 to 2020. Out of 1745 patients, 479 patients underwent NGS and 61 (12.7%) patients were identified as having an alteration in the PI3K pathway [132]. Patients harboring a co-altered *EGFR* was 8% of the study population. The authors concluded that PI3K

pathway alteration was more common in smoker male patients with NSCLC. Notably, this genetic aberration was not mutually exclusive to other mutations, thusly highlights the relationship between molecular pathways. Basically, given the potential importance of *PIK3CA* concomitant mutations molecular tumor boards are mandatory allowing for individualized therapy in this specific subset of patients. In summary, the limited sample size of these studies prevents us from drawing definitive conclusions. Larger studies with long term follow-up are warranted in order to clarify these controversial results.

5. Conclusions

While for decades NSCLC was considered to be a single disease, it is nowadays becoming more convenient to consider NSCLC as a combination of disease subtypes according to the driver genetic aberration. Concurrence of multiple driver alterations should be considered in order to comprehensively understand tumor mechanisms and therapeutic strategies. Currently, it is possible to identify a larger number of concomitant mutated cancers by using more sensitive and powerful techniques. Considering that the use of large panels of genes might induce to the identification of multiple targeted molecular drivers, multidisciplinary molecular tumor boards (MTBs) are mandatory in order to provide the best treatment strategies in cases of concurrent somatic genomic alterations [130–133]. On the other hand, the determination of the VAF, taking into account the number of cancer cells harboring concomitant genetic aberrations, might be used as a tool to select the correct therapeutic options for this particular subset of patients. In conclusion, co-existing driver gene alterations characterize a small group of NSCLC patients. However, further prospective studies are warranted to examinate the treatment outcomes of patients harboring double *EGFR* mutations.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jmp2020016/s1, Figure S1: PRISMA checklist; Figure S2: Search Strategy.

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