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The cell line models to study tyrosine kinase inhibitors in non-small cell lung cancer with mutations in the epidermal growth factor receptor: A scoping review

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ABSTRACT

Non-Small Cell Lung Cancer (NSCLC) represents ~85% of all lung cancers and ~15–20% of them are characterized by mutations affecting the Epidermal Growth Factor Receptor (EGFR). For several years now, a class of tyrosine kinase inhibitors was developed, targeting sensitive mutations affecting the EGFR (EGFR-TKIs). To date, the main burden of the TKIs employment is due to the onset of resistance mutations. This scoping review aims to resume the current situation about the cell line models employed for the in vitro evaluation of resistance mechanisms induced by EGFR-TKIs in oncogene-addicted NSCLC. Adenocarcinoma results the most studied NSCLC histotype with the H1650, H1975, HCC827 and PC9 mutated cell lines, while Gefitinib and Osimertinib the most investigated inhibitors. Overall, data collected frame the current advancement of this topic, showing a plethora of approaches pursued to overcome the TKIs resistance, from RNA-mediated strategies to the innovative combination therapies.

1. Introduction

Worldwide, lung cancers still represent one of the most incident cause of death, and according to GLOBOCAN 2020 it accounts for 11.4% of all new cancer cases, second only to breast cancer, but remains the leading cause of cancer mortality with the highest percentage of deaths, about 18%. In US, only in 2022, 236,740 new cases have been estimated, affecting 12% of men and 13% of women (Siegel et al., 2022; Sung et al., 2021). These data reflect the importance and the urgent need to reverse the course of this malignancy and slow down its impact.

Among preventable causes, tobacco smoking, secondhand smoke, alcohol use and HIV infections represent the main responsible for the onset of lung cancer, but on the other side family history, pulmonary fibrosis and carcinogenic chemicals exposure contribute to be the first causes in non-smoker patients (Alduais et al., 2023). The highest incidence of lung cancer has been registered in developing nations where cigarette smoking is more frequent, and not surprisingly, generally the tumorigenesis occurs after decades since the primary exposure, affecting people at least over 40 years of age. Conversely, when it occurs in younger people, especially in females, the main causes seem to be mainly associated with heritable mutations, rather than with environmental mutagen factors (Thandra et al., 2021).

1.1. Classification of lung cancers

Lung cancer is a heterogeneous disease from a molecular point of view, with an intra-tumor variety of cellular sub-populations with different features, like several other neoplasms (Herbst et al., 2018). The classification of this malignancy had been long based on morphology and immunohistochemistry. To date, the progress made with molecular

Abbreviations: ATP, Adenosine Triphosphate; Del, Deletion; EGFR, Epithelial Growth Factor Receptor; EMT, Epithelial Mesenchymal Transition; ErbB, Erythroblastic leukemia viral oncogene; FGFR, Fibroblast Growth Factor Receptor; HER, Human Epidermal Growth Factor Receptor; HSAEC, Human Small Airway Epithelial Cells; KDR, Kinase insert Domain Receptor; KRAS, Kristen Rat Sarcoma viral oncogene; LCLC, Large Cell Lung Cancer; MET, Mesenchymal Epithelial Transition; Met, Mesenchymal-epithelial transition factor; NOS, Not Otherwise Specified; NSCLC, Non-Small Cell Lung Cancer; SCC, Squamous Cell Carcinoma; SCLC, Small Cell Lung Cancer; TKI, Tyrosine-Kinase Inhibitor; VEGFR, Vascular Endothelial Growth Factor Receptor.

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features allows to classify non-small cell lung cancer (NSCLC) in oncogene addicted and non-oncogene addicted, thus driving the best treatment options. Nowadays, in fact, the main burden in the lung cancer context is given by NSCLC, rather than the less frequent small cell lung cancer (SCLC), also known as microcitoma. Among NSCLC, Lung Adenocarcinoma (AC or LUAD) and Lung Squamous Cell Carcinoma (SCC or LUSC) are two of the most representative variants, and hence the most mutations-affected NSCLC subtypes, followed by the Large Cell Lung Carcinoma (LCLC) and by a minority of poorly differentiated variants called "Not Otherwise Specified" (NOS) NSCLC (Nicholson et al., 2022).

1.2. Oncogene-addicted Non-Small Cell Lung Cancer (NSCLC): a cancer hallmark

The term "oncogene-addicted" referred to tumor means molecular aberrations affecting several components of signal transduction pathways involving the tumor growth and survival. Most of NSCLC can be reported also as oncogene-addicted NSCLC. In NSCLC these mutations can affect several targets as the ALK (anaplastic lymphoma kinase), MET (mesenchymal to epithelial transition), BRAF (v-raf murine sarcoma viral oncogene homolog B1), ROS1 (ROS proto-oncogene 1) KRAS (Kirsten rat sarcoma virus), and the EGFR (epidermal growth factor receptor). ALK rearrangement has been discovered recently, more in adenocarcinoma than in squamous cell carcinoma with highest percentage especially in advanced NSCLC stages, and especially in non-smoker patients. The EGFR is the most affected target accounting for a quarter of total NSCLC harboring mutations, and EGFR mutations constitute the 10–15% in patients with Caucasian ethnicity and 40% in east Asian patients of all NSCLC cases, rendering them good candidates for EGFR-TKIs (Paez et al., 2004). Not surprisingly, screening test for EGFR mutations is routinely performed to earlier identify and undertake the specific treatment (Calabrese et al., 2022; Smolle et al., 2020).

1.3. Epidermal Growth Factor Receptor (EGFR) and affecting mutations

EGFR is a transmembrane protein receptor, member of the tyrosine kinase receptors (RTKs) family. In particular, EGFR belongs to the HER/ErbB family composed of HER1 (ErbB1 or simply EGFR), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). It is activated by several ligands including the Epidermal Growth Factor (EGF). The dimerization and the autophosphorylation of the tyrosine residues of the intracellular domain, triggering the cytoplasmic signaling and hence the transcriptional processes aiding cell growth and survival as well as the inhibition of the apoptosis and therefore tumorigenesis (Xu et al., 2017).

Although EGFR is ubiquitously expressed in several sites, and exerts physiological functions, it may be implicated in the pathogenesis of numerous malignancies and associated with poor prognosis. 62% of NSCLC shows its overexpression correlating with a low survival rate, lymph node metastasis and reduced sensitivity to chemotherapy (Bethune et al., 2010; Sharma et al., 2007). This alteration seems to be due to i) the autocrine loop promoted by the ligands EGF and TNF-α highly expressed in NSCLC that leads to EGFR hyperactivity (Putnam et al., 1992; Rusch et al., 1993) or ii) the presence of several genetic aberrations that increase its expression. In fact, in different primary cancers it has been observed that EGFR can be affected by several mutations in the binding site of the RTK, more than it is in metastatic sites (Wei et al., 2014). These genetic mutations, besides being important as biomarkers, constitute relevant molecular targets. To date the most frequent sensitizing, and clinically relevant, EGFR mutations are present in the exons 18–21 encoding for the tyrosine kinase domain, and in some cases the presence of co-mutations in the same tumor may occur (Kobayashi and Mitsudomi, 2016). In the 90% of total cases, these co-mutations include the exon 19 deletions and the exon 21 L858R point mutation or "classic" mutations as opposed to those "rarest" or "uncommon" point mutations affecting exons 18 (G719C, G719S, G719A, V689M, N700D, E709K/Q, S720P mutations), exon 20 (V765A, S768I, V769L, T783A and T790M mutations/insertions) and exon 21 (N826S, S839T, K846R, L861Q, G863D mutations), or those aminoacidic insertions affecting only the exon 20 (Sharma et al., 2007).

1.4. EGFR-TKI and relative genotypic and phenotypic resistance mutations

Genetic changes in the EGFR coding gene consist of sensitizing mutations, which can be targeted by treatments with the EGFR tyrosine kinase inhibitor (EGFR-TKI) treatment. These inhibitors represent the gold standard for the advanced/metastatic NSCLC carrying these activating mutations, and all over the years several generations of TKI drugs were developed. The first generation, approved by the FDA from 2003 to 2011, was represented by a class of reversible competitive ATP-binding site molecules targeting the L858R and the exon 19 deletion affecting the EGFR. Gefitinib (ZD1839), Erlotinib (OSI-774) and Icotinib (BPI-2009 H) are among the most known and used, even if with different efficacy rate, and also with adverse effects. The main mechanisms of resistance to these TKIs consist of (i) the onset of secondary mutations, generally in-frame duplications or insertions (exon 20, T790M); (ii) phenotypic transformations in terms of histological shifts from adenocarcinoma to microcitoma (SCLC) or the acquisition of epithelial to mesenchymal transition (EMT); (iii) activation of alternative pathways representing the second more common cause of acquired resistance (Morgillo et al., 2013). Some years later, to overcome the first kind of resistance, second generation TKIs, mainly represented by Afatinib (BIBW 2992) and Dacomitinib (PF-00299804), were developed to cope additional mutations attributed to the HER family, in particular targeting HER2 and HER4, but this time with an irreversible covalent binding mode. However, the adverse effects produced by these drugs limited their application. The evolution of the most successful, still today, EGFR-TKI arrived only in 2015 with the Osimertinib (AZD9291) to counteract the resistance of the EGFR secondary mutation, T790M, sparing the wild-type RTK. Its selectivity against this mutation induced by 1st and 2nd generation TKIs is due to the formation of irreversible covalent bond with the C797 residue of the ATP-binding site of mutated EGFR. Its great potential is also due to the high clinical activity and tolerability, in addition to a long-term development of resistance mechanisms with respect to the previous ones. This 3rd generation EGFR-TKI targeting the T790M mutation, still represents the current standard of care continuing to record the highest PFS and OS (He et al., 2021; Herbst et al., 2018).

Together with EGFR genetic mutations, phenotypic changes can occur after TKI treatments. The histological evolution from Non-Small Cell Lung Cancer (NSCLC) to Small Cell Lung Cancer (SCLC) and the Epithelial Mesenchymal Transition (EMT) are the most frequent. While the onset of a SCLC could be favorable in terms of therapy options, the mesenchymal transition from an epithelial phenotype is not so auspicious as regards the treatment choices (Rolfo et al., 2014; Sequist et al., 2011).

In this context, the aim of this review was to collect the worldwide research performed to date on EGFR-mutated NSCLC cell line models treated with EGFR-TKIs in order to establish the current situation about this topic and to evaluate how these experimental models can be representative of the clinical applicability. Given the results obtained, we decided to analyze them performing a scoping review, aiming to report to date the global context about this issue.

2. Scoping review protocol

2.1. Information sources and search strategy

The search for articles was performed exclusively on PubMed entering the terms and combining them with Boolean operators (AND, OR) as follows: (NSCLC OR "non-small cell lung cancer") AND (EGFR OR

"epidermal growth factor receptor") AND (“cell line” OR “cell lines” OR “cell model”) AND (TKI OR "tyrosine kinase inhibitor"), for an advanced research limited to “title and abstract”.

2.2. Data items and collection process

Publication date was not restricted; all the results obtained by the research have been collected and classified according to the year of publication, journal, article type, experimental models employed. Regarding cell line models, classification was based on their name, tumor origin, the presence of possible EGFR mutations, the TKI used and the way these drugs were used, whether as single agent or in combination with other drugs. Once classified according to these parameters, the results have been selected or excluded for the analysis according to the eligibility criteria.

2.3. Study selection process

Data extraction was made considering the established eligibility criteria. According to the aim of this review, we decided to consider only the original articles dealing with at least one EGFR mutated NSCLC cell line, treated at least with one EGFR TKI. All the results that did not meet these criteria were excluded.

3. Results

3.1. Literature search and study characteristics

The search strategy employed produced 360 results, disseminated from January/February 1999 to March 2023, of which 344 original articles, 2 case reports, 11 reviews and 4 retracted articles, with a spread of publications registered especially from 2018 (Fig. 1).

The analysis of all the publications collected revealed 260 different types of cell lines employed, of which 156 belonging to lung cancers. From this classification it results that in vitro approach using cell lines is the most used model (69,36%), followed by animal models (mice) for the in vivo pre-clinical analyses (21,53%) and finally by human specimens (8,90%) for the ex vivo ones.

In order to deepen the features of the cell lines commonly employed, all those belonging to lung cancers and classified as NSCLC ones were analyzed. What emerged is a plethora of NSCLC histotypes, where the adenocarcinoma is the most represented among all (47%), followed by the squamous cell carcinoma (SCC) (19%) and other not otherwise specified cells (NOS) (19%), large cell lung carcinoma (LCLC) (7,5%), adenosquamous carcinoma (4,5%), and bronchioloalveolar (3%) subtype (Fig. 2).

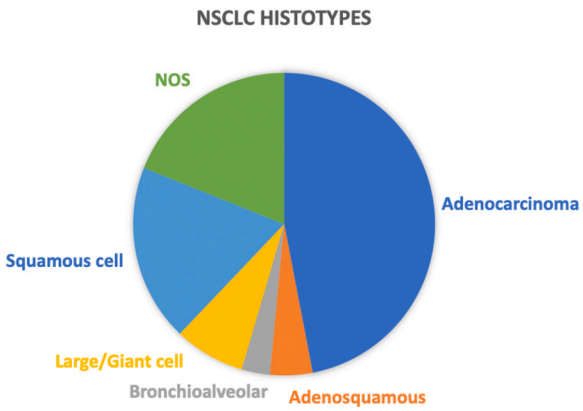

Fig. 2. : NSCLC histotypes: not otherwise specified non-small cell lung cancer (NOS); squamous cell carcinoma non-small cell lung cancer (Squamous cell); large and giant cell non-small cell lung cancer (Large/Giant cell); bronchioloalveolar non-small cell lung cancer (Bronchioloalveolar); adenosquamous non-small cell lung cancer (Adenosquamous) and adenocarcinoma non-small cell lung cancer (Adenocarcinoma).

3.2. Data extraction

In line with the aim of this scoping review, the citations retrieved by means of PubMed search were selected as reported in the flowchart represented in Fig. 3. Among all 360 results obtained, the papers that were not original articles and/or English-written were excluded. Of the remaining 339, 111 did not meet the inclusion criteria, therefore, 228 articles, dealing with at least one EGFR mutated NSCLC cell line, and treated with at least one EGFR TKIs have been considered.

3.3. Experimental cell line models of therapeutic strategies

All cell lines found in the selected articles have been classified according to the presence of EGFR mutations, excluding those where this aspect has not been specified. The adenocarcinoma histotype is the most represented among the EGFR mutated cell lines employed, variably represented by H1650, H1975, H3255, H820, HCC4006, HCC4011, HCC827 and PC-9 cell lines. These cells are characterized by several mutations affecting EGFR exons 18–21, the most frequent del E746-A750 of exon 19 and the L858R point mutation of exon 21, in addition to the resistance mutation T790M in the exon 20 found in H1975, H820 and PC-9 Gefitinib resistant cell line. Among the rarest ones found, many others related to exons 18, such as Y727C (Carlisle et al., 2007; Stabile et al., 2005; Thomas et al., 2005), and to exon 20, like R776C and

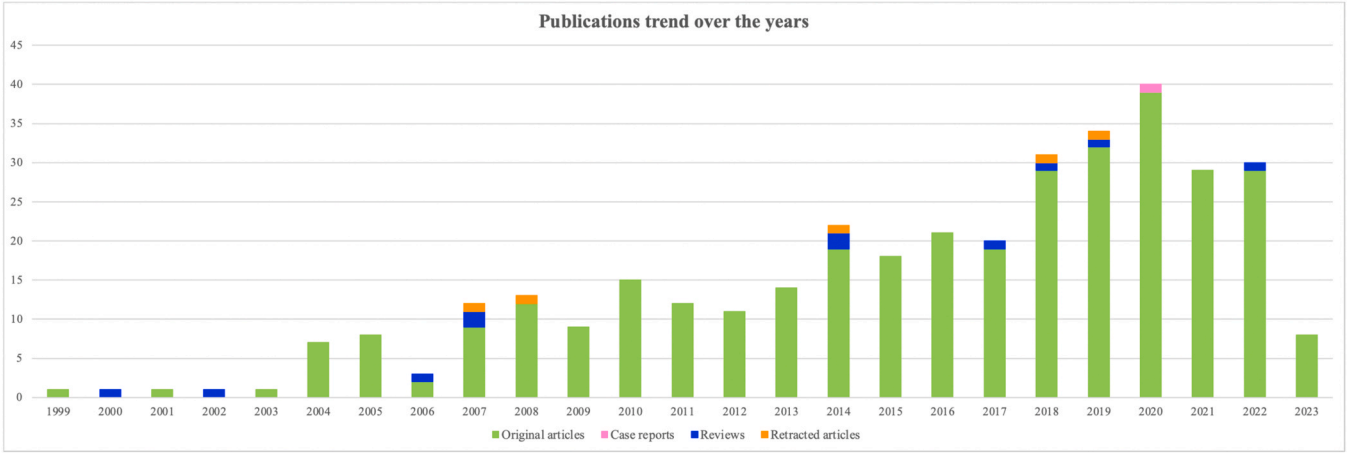

Fig. 1. : Publications trend from January/February 1999 to March 2023, representing the article types provided by the search strategy used.

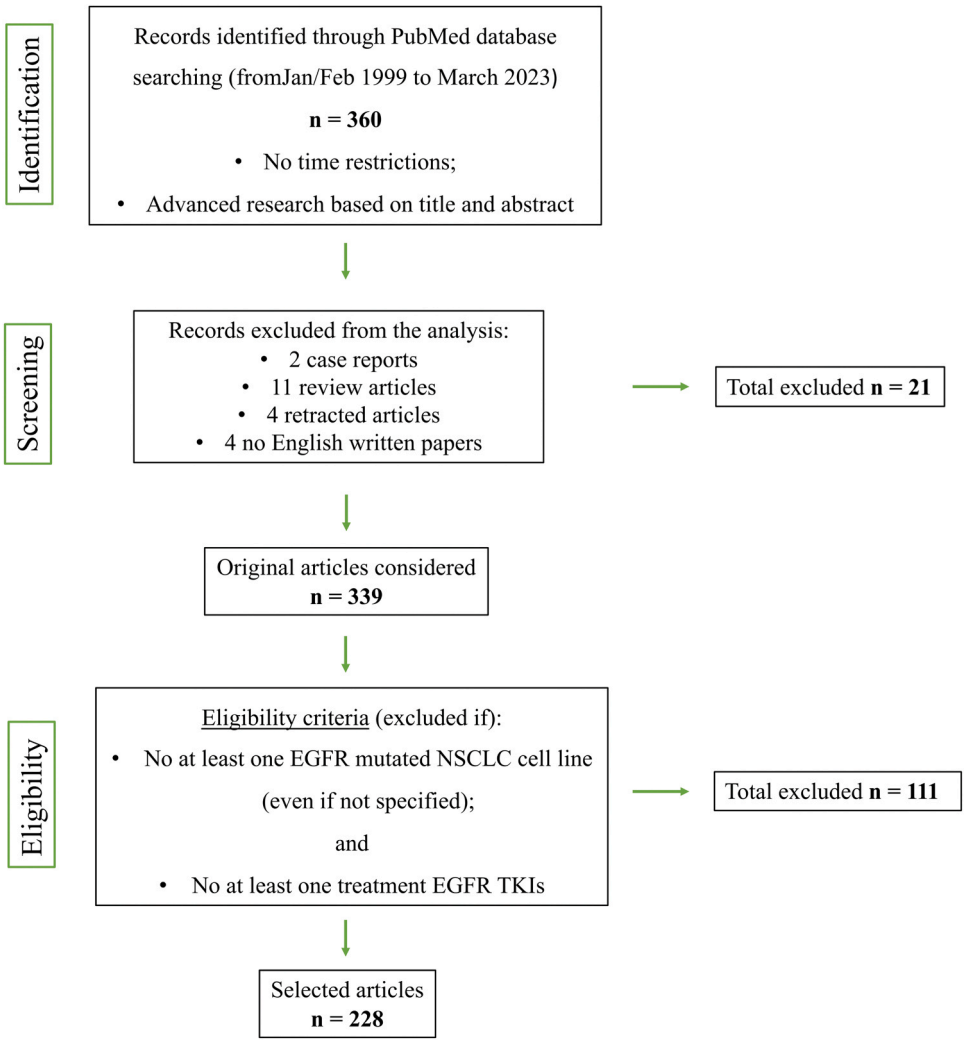

Fig. 3. Flowchart of article selection.

C797S (Shi et al., 2023; Wang and Yuang-Chi Chang, 2018). In some cases, many EGFR mutations coexist in the same cell line, like the exon 19 deletion and the resistance one in exon 20, without excluding many models in which it is possible to find not more than one mutation affecting another site different from EGFR. Table 1 resumes in detail all these results reporting specific information about each cell line investigated. The H3255 cell line results the most cited among the ones harboring the primary L858R mutated cell lines, often employed in several analyses together with others with different genetic arrangements (Gandhi et al., 2009; Helfrich et al., 2006; Kobayashi et al., 2012; Martin et al., 2016; Ohara et al., 2021; Sudo et al., 2013; Tsai et al., 2013).

The added value of H1975, characterized by the secondary resistance mutation, is due also to the on-target L858R and the off-target mutation related to p53, that for the most are employed to study Osimertinib and related resistance (X. Li et al., 2021; Nanamiya et al., 2021; Wang et al., 2023). Makimoto and colleagues (Makimoto et al., 2021), dealing with Osimertinib resistance, experienced an innovative lung adenocarcinoma cell line, establishing ABC-31 with activated IGF1R as a new potential model strategy against Osimertinib resistance. Among the less mutated known and the less used cell line models, a peculiar mutation profile was found in lung adenocarcinoma cell line H4006, that according to American Type Culture Collection (ATCC) is characterized by the exon 19 L747-E749 deletion, A750P.

3.4. EGFR-TKIs used in cell line models

The analysis revealed that more than a half of all TKIs investigated are EGFR-TKIs, some of them with multitarget potential and, hence, effective also against other targets, like VEGFR, HDAC, Abl, Src, c-Kit, HER2, MAPK and JAK3, just to name a few. Table 2 resumes all of them based on the molecular target (if they are multitarget) and on the target binding mode. However, consisting with the aim of this review, we mainly focused on those acting against EGFR. These inhibitors are usually classified as belonging to first, second, third or the most recent fourth TKI generation, according to their specific features. The most employed EGFR-TKIs in vitro are Afatinib, Erlotinib, Gefitinib, Icotinib and Osimertinib, considering that this latter has been approved recently with respect to the others. In many cases, the cell lines used were already employed as TKI-resistant, in some others the resistance was induced in vitro by maintaining cells in culture with different doses of TKI. Several studies adopted the TKIs not only as a single treatment to induce or to study the behind resistance mechanisms, but also in combination among them or with other drugs or other treatment strategies, including chemotherapy, natural compounds, targeted strategies by using RNA molecules, antibodies or radiation treatment.

Obviously, the works dated before 2015 have led to demonstrate the efficacy of the current treatment standard in several mutated cell lines, that could appear outdated to date. What emerged from this analysis is the assessment of TKIs regulated molecular mechanisms involved in

Table 1
EGFR mutated cell lines collected and reporting according to the relative NSCLC histotype, specific sensitivity or resistance EGFR mutations, the exon(s) affected by the genetic rearrangements.

NSCLC cell lines	Origin histotype	EGFR mutations	Exon (s) affected by EGFR mutations	Other mutations or considerations	References
ABC-31	*Adenocarcinoma*	*L858R*	*exon 21*	*T790M and C797S lost*	(Makimoto et al., 2021)
11_18	*Adenocarcinoma*	*L858R*	*exon 21*		(Banno et al., 2015; Liu et al., 2021)
273 T	*Squamous Cell Carcinoma*	*Y727C*	*exon 18*		(Carlisle et al., 2007; Stabile et al., 2005; Thomas et al., 2005)
As13	*NOS*	*R776C + L858R*	*exons 20 and 21*		(Wang and Yuang-Chi Chang, 2018)
H1650	*Adenocarcinoma*	*Del E746-A750*	*exon 19*	*PTEN del -/-*	(Alama et al., 2015; Cho et al., 2011; Cragg et al., 2007; Dong et al., 2012, 2020; Dowlati et al., 2008; Fei et al., 2013; Gandhi et al., 2009; Gilmer et al., 2008; Giovannetti et al., 2010, 2008; Han et al., 2020; Helfrich et al., 2006; Hong et al., 2017; Hu et al., 2017; Hülsmann et al., 2014; Ito et al., 2014; Kim et al., 2013; Ko et al., 2009, 2008; Kobayashi et al., 2012; Lee et al., 2011; Leung et al., 2016; K.Li et al., 2019;Lin et al., 2017;Loriot et al., 2010;Martin et al., 2016;Moody et al., 2017;Nakachi et al., 2010;Nam et al., 2016;Park et al., 2019; Peled et al., 2013a, 2013b;Sahin et al., 2021;Shen et al., 2010; J. M. Song et al., 2018; X.Song et al., 2018;Sudo et al., 2013; F.Wang et al., 2020;Wang and Yuang-Chi Chang, 2018;Wang et al., 2014;Xu et al., 2012;Yang et al., 2015;Yao et al., 2010; H.Zhang et al., 2019; N.Zhang et al., 2019;Zhang and Wang, 2018)
H1975	*Adenocarcinoma*	*L858R and T790M*	*exons 21 and 20*	*p53 mutation*	(Alama et al., 2015; Ao et al., 2022; Bokobza et al., 2014; Cao et al., 2017; J.-C.Chen et al., 2019; T.Chen et al., 2019; Chen et al., 2018; Cheng et al., 2011;Choi et al., 2007;Costa et al., 2007;Cragg et al., 2007;Dai et al., 2016;Dong et al., 2015, 2012, 2020;Dowlati et al., 2008;Fawwaz et al., 2021, 2020;Fei et al., 2013;Freeman et al., 2009; Gandhi et al., 2009;Gilmer et al., 2008;Giovannetti et al., 2010;Han et al., 2020;Hasako et al., 2018;Helfrich et al., 2006;Hong et al., 2017; Hsieh et al., 2018;Hsu et al., 2016;Hu et al., 2020, 2016, 2017;Ito et al., 2014;Iwai et al., 2012;Jang et al., 2016;Ji et al., 2020; J. H.Kim et al., 2021; S.Kim et al., 2021;Kim et al., 2023;Kobayashi et al., 2012; Kommineni et al., 2021;Kosibaty et al., 2022;La Monica et al., 2020; Lee et al., 2011, 2022;Lei et al., 2023;Leung et al., 2016;Li et al., 2022, 2017; J.Li et al., 2013a; K.Li et al., 2019; X.-Y.Li et al., 2019; X.Li et al., 2021, 2019; Y.-Y.Li et al., 2013;Lim et al., 2022;Lin et al., 2017; Y.-N. Liu et al., 2019; Y.Liu et al., 2019;Lopez Sambrooks et al., 2018;Loriot et al., 2010;Luan et al., 2022;Makimoto et al., 2021;Martin et al., 2016; Moody et al., 2017;Morgillo et al., 2013;Nakachi et al., 2010;Nam et al., 2016;Nanamiya et al., 2021;Narita et al., 2017;Nigro et al., 2019;Nishihara et al., 2022;Nishiya et al., 2015;Ochi et al., 2020; Ohara et al., 2021;Ohtsuka et al., 2010;Osude et al., 2022;Park et al., 2022;Peled et al., 2013a, 2013b;Qin et al., 2021, 2020;Qu et al., 2021; Quick, 2023;Raoof et al., 2019;Shi et al., 2023; J. M.Song et al., 2018; Sos et al., 2008;Soucheray et al., 2015;Sudo et al., 2013;Sun et al., 2020;Tang et al., 2016;Terasaki et al., 2019;Tominaga et al., 2016; Tung et al., 2017;Umeda et al., 2017;Wang et al., 2015, 2023, 2014; F. Wang et al., 2020;Wang and Yuang-Chi Chang, 2018; T.-H.Wang et al., 2020;Watanabe et al., 2021;Xu et al., 2016, 2012;Yang et al., 2015;Yao et al., 2010; H.Zhang et al., 2019; N.Zhang et al., 2019;Zhang and Wang, 2018; M.Zhao et al., 2018;Zhao et al., 2016;Zhen et al., 2015; Zhou et al., 2021)
H3255	*Adenocarcinoma*	*L858R*	*exon 21*		(Chiang et al., 2018; Choi et al., 2007; Costa et al., 2007; Cragg et al., 2007; Fawwaz et al., 2021, 2020; Gandhi et al., 2009; Giovannetti et al., 2010; Helfrich et al., 2006; Kim et al., 2013; Kobayashi et al., 2012; Moreira-Leite et al., 2010; Nishiyama et al., 2020; Ohara et al., 2021; Osude et al., 2022; Saleem et al., 2011; Sos et al., 2008; Tsai et al., 2013, 2011; Umeda et al., 2017; Yang et al., 2007; Yasuda et al., 2012; Yonesaka et al., 2008; Zhang et al., 2008; Zhen et al., 2015)
H4006	*Adenocarcinoma*	*Del L747-E749, A750P*	*exon 19*		(Choi et al., 2007; Liu et al., 2021)
H820	*Adenocarcinoma*	*Del E746–749, T790M*	*exons 19 and 20*	*c-Met amplification*	(Dong et al., 2020; Gandhi et al., 2009; Helfrich et al., 2006; Kobayashi et al., 2012; Leung et al., 2016; Martin et al., 2016; Sudo et al., 2013; Tsai et al., 2013, 2011; Wang and Yuang-Chi Chang, 2018)
HCC2279	*Adenosquamous cell carcinoma*	*Del E746-A750*	*exon 19*		(Choi et al., 2007; Gandhi et al., 2009; Kim et al., 2013; Peled et al., 2013a; Sudo et al., 2013; Zhang et al., 2008)
HCC2935	*Adenocarcinoma*	*Del E746-A751*	*exon 19*		(Dong et al., 2020; Kobayashi et al., 2012; Martin et al., 2016; R. Nakamura et al., 2023; T.Nakamura et al., 2023;Sudo et al., 2013)
HCC4006	*Adenocarcinoma*	*Del E746-A750*	*exon 19*		(Chiu et al., 2020; Gandhi et al., 2009; Haque et al., 2020; Hashida et al., 2015; Helfrich et al., 2006; Kurihara et al., 2019; Y.-Y.Li et al., 2013;Lim et al., 2022; Y.-N.Liu et al., 2019; Luan et al., 2022; R. Nakamura et al., 2023; T.Nakamura et al., 2023;Ohara et al., 2021; Peled et al., 2013b;Raoof et al., 2019;Soucheray et al., 2015;Stewart

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Table 1 (*continued*)

NSCLC cell lines	Origin histotype	EGFR mutations	Exon (s) affected by EGFR mutations	Other mutations or considerations	References
					et al., 2021;Sudo et al., 2013;Umeda et al., 2017;Yang et al., 2007;Yao et al., 2010;Yonesaka et al., 2008;Zhang et al., 2018)
HCC4011	*Adenocarcinoma*	*L858R*	*exon 21*		(Baumgartner et al., 2018; Chiang et al., 2018; Gandhi et al., 2009; Hashida et al., 2015; Kobayashi et al., 2012; Kurihara et al., 2019)
HCC827	*Adenocarcinoma*	*Del E746–A750*	*exon 19*		(Ahn et al., 2014; Ao et al., 2022; Baldacci et al., 2018; Banno et al., 2015; Baumgartner et al., 2018; Bokobza et al., 2014; Cao et al., 2017; Cappuzzo et al., 2009; J.Chen et al., 2020; T.Chen et al., 2019; Z.Chen et al., 2022;Cheong et al., 2018;Choi et al., 2007;Clement et al., 2020; Costa et al., 2007;Cragg et al., 2007;Dai et al., 2021, 2016;Dong et al., 2015, 2012;Galetti et al., 2015;Hasako et al., 2018;Hashida et al., 2015;He et al., 2020;Helfrich et al., 2006;Hsu et al., 2016;Hu et al., 2020;Imabayashi et al., 2019;Iwai et al., 2012;Jang et al., 2016;Ji et al., 2020;Kim et al., 2020, 2013; S.Kim et al., 2021;Kobayashi et al., 2012;Kommineni et al., 2021;Kosibaty et al., 2022;La Monica et al., 2020, 2019a, 2019b; A.-F.Lee et al., 2017; Lee et al., 2022; K. E.Lee et al., 2017;Lei et al., 2023;Leung et al., 2016;Li et al., 2017, 2020; K.Li et al., 2019; X.Li et al., 2019; Y.-Y.Li et al., 2013;Lim et al., 2022;Liu et al., 2021, 2018;Lou et al., 2019;Martin et al., 2016;Moreira-Leite et al., 2010; T.Nakamura et al., 2023;Narita et al., 2017;Nigro et al., 2019;Ninomiya et al., 2018;Ochi et al., 2020;Ohara et al., 2021;Peng et al., 2021;Pool et al., 2017;Qi et al., 2018;Raoof et al., 2019;Sahin et al., 2021;Sano et al., 2015;Slobbe et al., 2014; J. M.Song et al., 2018; Soucheray et al., 2015;Stewart et al., 2021;Sudo et al., 2013;Tang et al., 2018;Terp et al., 2021;Tsai et al., 2013, 2011;Umeda et al., 2017; Wang et al., 2018, 2014, 2019;Wang and Yuang-Chi Chang, 2018; Watanabe et al., 2021;Wen et al., 2020;Wu et al., 2022;Yang et al., 2015, 2012;Yao et al., 2010;Yonesaka et al., 2008;Yu et al., 2020, 2016;Zhang et al., 2008, 2016; N.Zhang et al., 2019; T.Zhang et al., 2019; Zhen et al., 2015;Zhou et al., 2021; L.Zhu et al., 2021; X.Zhu et al., 2021)
LLC	*Squamous Cell Carcinoma*	*Del E746-A750, T790M + C797S*	*exons 19 and 20*		(Shi et al., 2023)
MGH174-2A	*NOS*	*Del E746-A750*	*exon 19*		(Raoof et al., 2019)
MGH700-2D	*NOS*	*Del E746-A751*	*exon 19*		(Raoof et al., 2019)
MGH707-1	*NOS*	*Del E746-A750, T790M*	*exons 19 and 20*		(Raoof et al., 2019)
MGH721-1	*NOS*	*Del E746-A750, T790M*	*exons 19 and 20*		(Raoof et al., 2019)
MGH792-1A	*NOS*	*L858R*	*exon 21*		(Raoof et al., 2019)
PC-14	*Adenocarcinoma*	*Del E746-A750, T790M*	*exons 19 and 20*		(Arao et al., 2004; Kitamura et al., 2014; Ohtsuka et al., 2010)
PC-9	*Adenocarcinoma*	*Del E746-A750*	*exon 19*	*Gefitinib sensitive*	(An et al., 2023; Ao et al., 2022; Arai et al., 2020; Arao et al., 2004; Banno et al., 2015; Baumgartner et al., 2018; Bokobza et al., 2014; Cai et al., 2021; Cao et al., 2017; H.Chen et al., 2022; Z.Chen et al., 2022; Cheng et al., 2011;Chiang et al., 2018;Choi et al., 2015;Costa et al., 2007;Dai et al., 2016;Ding et al., 2022;Dong et al., 2012;Fei et al., 2013;Gandhi et al., 2009;Gao et al., 2014;Guardiola et al., 2022;Han et al., 2020;Hashida et al., 2015;Hirata et al., 2005;Hou et al., 2019; Huang et al., 2022, 2020;Ichihara et al., 2009;Imabayashi et al., 2019; Isozaki et al., 2016;Jang et al., 2016;Ji et al., 2020;Ju et al., 2010;Kato et al., 2018;Kim et al., 2020, 2013; J. H.Kim et al., 2021;Kitamura et al., 2014;Kobayashi et al., 2012;Koizumi et al., 2005;Kurihara et al., 2019;La Monica et al., 2020, 2019b, 2019a; A.-F.Lee et al., 2017; Lee et al., 2022, 2016;Li et al., 2014, 2015, 2020; J.Li et al., 2013b; Q.Li et al., 2021; X.-Y.Li et al., 2019; X.Li et al., 2019;Lim et al., 2022;Liu et al., 2015, 2021; Y.-N.Liu et al., 2019; Y.Liu et al., 2019;Lopez Sambrooks et al., 2018;Makimoto et al., 2021;Martin et al., 2016;Nam et al., 2016;Nanamiya et al., 2021;Nigro et al., 2019;Ninomiya et al., 2018;Nishihara et al., 2022;Nishiyama et al., 2020;Nukatsuka et al., 2012;Ochi et al., 2014;Ogino et al., 2007;Ohara et al., 2021;Park et al., 2022, 2019;Plangger et al., 2022;Qi et al., 2018;Raoof et al., 2019; Sahin et al., 2021;Sano et al., 2015;Serizawa et al., 2014;Shao et al., 2018;Shien et al., 2014; X.Song et al., 2018;Stewart et al., 2021;Sudo et al., 2013;Sun et al., 2020;Takeuchi et al., 2009;Tanino et al., 2018; Taron et al., 2005;Terp et al., 2021;Togashi et al., 2015;Tominaga et al., 2016;Tong et al., 2019;Tsai et al., 2013, 2011;Wang et al., 2018, 2013, 2019, 2021;Watanabe et al., 2021;Wen et al., 2020;Wu et al., 2014, 2020;Xu et al., 2012;Yamaoka et al., 2017, 2016;Yang et al., 2012;Yao et al., 2010;Yeo et al., 2022;Yonesaka et al., 2008;Zhang et al., 2018; N.Zhang et al., 2019; T.Zhang et al., 2019; H.Zhao et al., 2018; M.Zhao et al., 2018;Zhao et al., 2016; L.Zhu et al., 2021)

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Table 1 (*continued*)

NSCLC cell lines	Origin histotype	EGFR mutations	Exon (s) affected by EGFR mutations	Other mutations or considerations	References
PE089	*Adenocarcinoma*	*Del E746-A750, T790M*	*exons 19 and 20*		(Hsiao et al., 2020; Yang et al., 2015)

proliferation, migration and cytotoxicity. To evaluate the resistance mechanisms induced by TKIs, wild-type and EGFR mutated models were often compared, and siRNA-mediated knockdown methodologies were frequently studied. (Choi et al., 2007; Costa et al., 2007; Yang et al., 2012). The need to deepen the outcomes caused by EGFR mutations has pushed to set up combinational approaches between TKIs, or several other inhibitors, to increase the sensitivity of cells through an additive effect (Arao et al., 2004; Thomas et al., 2005). Due to its earlier approval, Gefitinib resulted the most investigated one still to date. In vitro studies revealed really important to demonstrate its great efficacy that still today makes it the gold standard as NSCLC first-line treatment. This is because of EGFR is the one of the main target considered in the NSCLC adenocarcinomas treatment, that due to the presence of EGFR mutations make EGFR-TKIs the first-line therapy persecuted (Taron et al., 2005).

Some interesting studies enhanced the inhibitors using radiolabeled TKIs, e.g. Afatinib, to evaluate the development of acquired resistance mechanism such as the EMT and stem-cell-like features (Hashida et al., 2015; Slobbe et al., 2014).

With the advent of Osimertinib, that since its approval recorded great clinical efficacy, the scientific literature production showed several studies based on the evaluation of its associated resistance mechanism (La Monica et al., 2020, 2019a, 2019b; Martin et al., 2016; Tang et al., 2018, 2016).

In most cases, the synergistic support targeting other tyrosine-kinase receptors overcomes the resistance induced by other TKIs, as with the reversible multitarget AE788 (Nakachi et al., 2010), or with the BMS-690514 and the T790M-specific HS-10182 that can represent successful adjuvants to clinical radiotherapy (Chen et al., 2018; Loriot et al., 2010). Among the other TKIs encountered, some of them played a minor role in the investigation, such as the multitarget Foretinib that was used in a wide context of Osimertinib resistant cells, investigating other underlying mechanisms involving other pathways (Nishiyama et al., 2020).

Among the third generation TKIs, Naquotinib, Rociletinib, TAS-121 and WZ4002 were investigated. The latter was developed against T790M resistance mutation, and also against the EMT-associated resistance (Ji et al., 2020; Raoof et al., 2019; Umeda et al., 2017). Thyrophostin AG1478 was studied in terms of growth-inhibitory effects in combination with Afatinib (Banno et al., 2015), a similar approach used for the employment of Genistein that was the only natural-derived TKI observed (Koizumi et al., 2005). Vandetanib, instead, was employed to induce resistance after chronic exposure in cells harboring the secondary T790M to deepen the related resistance mechanism (Ichihara et al., 2009).

The pan-EGFR mutation selective TAS6417, also known as Zipalertinib, showed great efficacy against EGFR exon 20 mutated models, more than in WT ones, supporting the positive results and tolerability observed in the clinical setting (Hasako et al., 2018).

3.5. *In vitro strategies to study resistance mechanisms*

The wide variability of the research work performed and collected showed different approaches to study the resistance mechanisms. EGFR mutated cell line models have been used for different purposes before and after the in vitro induction of resistance, by using different protocols (Lee et al., 2016). The same cell line has been used for different purposes, contributing to analyze the resistance mechanism related to different TKIs from different perspectives and with different strategies and molecular approaches. In the following paragraphs, the most cited approaches to counteract the EGFR-TKIs resistance are reported.

3.5.1. *Genetic insights in structural and mutational profiles in EGFR*

To investigate the mechanisms related to the resistance induced after TKIs administration it was focused on the issues related to the EGFR itself. Among all the results collected, some authors, in fact, have shed light on genes until now not associated with EGFR-mutant NSCLC, performing their studies on the HCC827 and HCC2279 cell lines, affected respectively by exon 19 deletion, and on the H3255 and H1975 harboring the L858R one. They decided to assess the transcriptional profile of the activating EGFR somatic mutations affecting many cell line models, revealing clinical significance of two of them involved in enhancing the clonogenicity of these cells, finding potential therapeutic targets (Choi et al., 2007). Even if with different aims, HCC827, and the other mutational profiles carried by PC9, H4006 and 11_18 were employed to predict the sensitivity to Osimertinib, by performing an in situ proximity ligation assay (PLA) technique, a method to identify physical closeness of proteins. This method, detecting the interaction between the homo- or heterodimerization of Her1 (EGFR), was able to establish the ratio of heterodimers to homodimers finding a significant positive correlation with the IC50 of Osimertinib, suggesting that this ratio is a potential predictor of EGFR-TKI sensitivity (Liu et al., 2021). The sensitivity to specific drug seems to be increased by the presence of in-frame deletion. To demonstrate this, PC9 NSCLC cells have been chosen and employed to compare the sensitivity to ZD6474, a vascular endothelial growth factor inhibitor, with that obtained by using Gefitinib. What emerged is that PC9 cells containing a 15-bp in-frame EGFR deletion may confer increased sensitivity both to ZD6474, an inhibitor of VEGFR-2 tyrosine kinase, and gefitinib (Arao et al., 2004). Given the high sensitivity to Gefitinib, PC9 cell line has been employed for the most as a model to study its resistance mechanisms. RPC9 (Resistant PC9) was established as a Gefitinib resistant model to study the acquired resistance in NSCLC, demonstrating that the T790M mutation, which occurs in EGFR exon 19 deletion, is linked to the increase of resistance, and hence with cell growth. Therefore, the authors demonstrated the reversion of TKI resistance by introducing EGFR carrying the activating deletion into RPC-9 cells, highlighting the relationship underlying the Gefitinib sensitivity, but in this case depending on the balancing of sensitive and resistant EGFR alleles (Ogino et al., 2007).

3.5.2. *Biomarkers detection and alternative inhibitory strategies*

The acquired resistance of TKIs has been often correlated with important biomarkers, involved in several pathways and often useful as potential therapeutic target. CD82 is one of these. It deals with a cancer metastatic suppressor protein often correlated with good prognosis of NSCLC: in fact, it is up-regulated in wild-type EGFR but down-regulated in mutated ones. PE089 cell line, harboring T790M mutation, was used to evaluate the correlation between the overexpression of EGFR mutated and the downregulation of the tumor suppressor CD82. What emerged from this analysis is promising for the treatment of NSCLC adenocarcinoma, because it was demonstrated that when mutated EGFR is overexpressed, the CD82 level decreases with respect to the wild-type EGFR, suggesting a stronger correlation between them. These suggest that the up-regulation of CD82 can represent a possible treatment strategy to suppress the tumorigenic potential of EGFR mutated (Yang et al., 2015). In HCC827 Gefitinib-resistant clones, instead, other molecular markers

Table 2
Report of EGFR-TKIs and multitarget TKIs considered, classified according to the molecular target(s) and relative binding mode.

TKI name	Molecular target	TKI target binding mode	Other considerations	References
AEE788	HER1/2, VEGFR, EGFR, ErbB2, KDR, Fit	Reversible		(Nakachi et al., 2010)
Afatinib	EGFR (ErbB1), HER2 (ErbB2), ErbB3 and ErbB4	Irreversible		(Alama et al., 2015; An et al., 2023; Banno et al., 2015; Baumgartner et al., 2018; Dai et al., 2021; Estrada-Bernal et al., 2021; Floc'h et al., 2020, 2018; Hasako et al., 2018; Hashida et al., 2015; Hoshi et al., 2017; Hu et al., 2017; Huo et al., 2022; Imabayashi et al., 2019; Leung et al., 2016; Li et al., 2020; Liu et al., 2021; Y.-N.Liu et al., 2019; Lopez Sambrooks et al., 2018;Makimoto et al., 2021; T. Nakamura et al., 2023;Ninomiya et al., 2018;Ohara et al., 2021; Plangger et al., 2022;Slobbe et al., 2014;Tang et al., 2016;Wang and Yuang-Chi Chang, 2018;Wang et al., 2019;Yamaguchi et al., 2014; X.Zhu et al., 2021)
BMS-690514	VEGFR and EGFR (HER2, HER4)	Reversible		(Loriot et al., 2010)
Erlotinib	EGFR	Reversible		(An et al., 2023; J.-C.Chen et al., 2019;Cheong et al., 2018;Chiang et al., 2018;Cho et al., 2011;Choi et al., 2007;Clement et al., 2020; Costa et al., 2007;Dai et al., 2016;Dong et al., 2020;Gandhi et al., 2009;Giovannetti et al., 2008;Haque et al., 2020;Hasako et al., 2018; He et al., 2020;Hong et al., 2017;Hsu et al., 2016;Hülsmann et al., 2014;Imabayashi et al., 2019;Isozaki et al., 2016;Ito et al., 2014;Iwai et al., 2012;Kim et al., 2020; J. H.Kim et al., 2021; S.Kim et al., 2021; Ko et al., 2009;Kobayashi et al., 2012;Kommineni et al., 2021; X.Li et al., 2019; Y.-Y.Li et al., 2013;Li et al., 2020;Liu et al., 2021; Y.-N. Liu et al., 2019;Lopez Sambrooks et al., 2018;Makimoto et al., 2021; Nakachi et al., 2010;Nam et al., 2016;Nanamiya et al., 2021; Nishiyama et al., 2020;Nukatsuka et al., 2012;Osude et al., 2022; Park et al., 2022, 2019;Pool et al., 2017;Sahin et al., 2021;Sano et al., 2015;Serizawa et al., 2014;Shao et al., 2018; J. M.Song et al., 2018; Soucheray et al., 2015;Stewart et al., 2021;Tang et al., 2016; Tanino et al., 2018;Terp et al., 2021;Tung et al., 2017;Watanabe et al., 2021;Yang et al., 2007;Yao et al., 2010;Yasuda et al., 2012;Yu et al., 2016;Zhang et al., 2016;Zhou et al., 2021; L.Zhu et al., 2021)
Foretinib	HGFR and VEGFR, c-Met and KDR. (Little activity to FGFR1 and EGFR)	ATP competitive inhibitor		(Nishiyama et al., 2020)
Gefitinib	EGFR	Reversible		(Ahn et al., 2014; An et al., 2023; Arai et al., 2020; Arao et al., 2004; Baldacci et al., 2018; Baumgartner et al., 2018; Bokobza et al., 2014; Cai et al., 2021; Cao et al., 2017; Cappuzzo et al., 2009; Carlisle et al., 2007; H.Chen et al., 2022; J.Chen et al., 2020;Cheng et al., 2011;Cheong et al., 2018;Chiu et al., 2020;Choi et al., 2015, 2007; Costa et al., 2007;Cragg et al., 2007;Ding et al., 2022;Dong et al., 2015, 2012;Fawwaz et al., 2020;Fei et al., 2013;Galetti et al., 2015; Gandhi et al., 2009;Gao et al., 2014;Gilmer et al., 2008;Giovannetti et al., 2010;Guardiola et al., 2022;Han et al., 2020;Helfrich et al., 2006;Hirata et al., 2005;Hou et al., 2019;Hsiao et al., 2020;Hsieh et al., 2018;Hu et al., 2020, 2016;Huang et al., 2022;Ichihara et al., 2009;Jang et al., 2016;Ju et al., 2010;Kato et al., 2018;Kim et al., 2013; J. H.Kim et al., 2021; S.Kim et al., 2021;Kitamura et al., 2014; Ko et al., 2008;Kobayashi et al., 2012;Koizumi et al., 2005;Kosibaty et al., 2022;Kurihara et al., 2019;La Monica et al., 2020; A.-F.Lee et al., 2017;Lee et al., 2011, 2022, 2016; K. E.Lee et al., 2017;Li et al., 2014, 2017, 2015; Q.Li et al., 2021; X.-Y.Li et al., 2019;Lin et al., 2017;Liu et al., 2015, 2018; Y.-N.Liu et al., 2019;Lopez Sambrooks et al., 2018;Lou et al., 2019;Luan et al., 2022;Makimoto et al., 2021;Martin et al., 2016;Moody et al., 2017;Moreira-Leite et al., 2010;Morgillo et al., 2013; T.Nakamura et al., 2023;Nam et al., 2016;Nanamiya et al., 2021;Narita et al., 2017;Nigro et al., 2019;Ninomiya et al., 2018;Nishihara et al., 2022;Nishiya et al., 2015;Nishiyama et al., 2020;Ochi et al., 2014;Ogino et al., 2007; Peled et al., 2013b;Peng et al., 2021;Qi et al., 2018;Raoof et al., 2019;Saleem et al., 2011;Sano et al., 2015;Serizawa et al., 2014; Shen et al., 2010; X.Song et al., 2018;Sos et al., 2008;Soucheray et al., 2015;Stabile et al., 2005;Stewart et al., 2021;Sudo et al., 2013; Tang et al., 2016;Taron et al., 2005;Terasaki et al., 2019;Terp et al., 2021;Thomas et al., 2005;Togashi et al., 2015;Tominaga et al., 2016;Tsai et al., 2013, 2011;Umeda et al., 2017;Wang et al., 2015, 2018, 2014, 2019, 2021; F.Wang et al., 2020;Wang and Yuang-Chi Chang, 2018;Wen et al., 2020;Wu et al., 2022, 2014, 2020;Xu et al., 2016, 2012;Yamaoka et al., 2017, 2016;Yang et al., 2015;Yeo et al., 2022;Yonesaka et al., 2008;Zhang et al., 2018, 2008, 2020; N.Zhang et al., 2019; T.Zhang et al., 2019;Zhang and Wang, 2018; H.Zhao et al., 2018; M.Zhao et al., 2018;Zhao et al., 2016;Zhen et al., 2015)

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Table 2 (*continued*)

TKI name	Molecular target	TKI target binding mode	Other considerations	References
Genistein	protein-tyrosine kinase and topoisomerase-II	*wide mechanism, several pathways involved*	An isoflavonoid derived from soy products	(Koizumi et al., 2005)
HS-10182	EGFR with T790 mutation	*not specified*		(Chen et al., 2018)
Icotinib	EGFR	Reversible		(An et al., 2023; Ao et al., 2022; Huang et al., 2020; K.Li et al., 2019; Wang et al., 2018;Yang et al., 2012; N.Zhang et al., 2019; T.Zhang et al., 2019)
Lapatinib	EGFR (Erb1) e HER2/neu (ErbB2)	ATP competitive inhibitor		(Dowlati et al., 2008; Gilmer et al., 2008; Moody et al., 2017)
Naquotinib	EGFR with T790 mutation	Irreversible		(Ninomiya et al., 2018)
Osimertinib	EGFR with T790 and L858R mutation (exon 19 and exon 21)	Irreversible		(An et al., 2023; Cai et al., 2021; T.Chen et al., 2019; Z.Chen et al., 2022;Guardiola et al., 2022;Han et al., 2020;Hirata et al., 2005; Imabayashi et al., 2019;Ji et al., 2020; S.Kim et al., 2021;Kommineni et al., 2021;Kosibaty et al., 2022;La Monica et al., 2020, 2019b, 2019a;Lei et al., 2023;Li et al., 2022, 2020; X.-Y.Li et al., 2019; X.Li et al., 2021;Lim et al., 2022;Lin et al., 2017;Liu et al., 2021; Y.Liu et al., 2019;Lopez Sambrooks et al., 2018;Luan et al., 2022; Makimoto et al., 2021;Martin et al., 2016;Nanamiya et al., 2021; Ninomiya et al., 2018;Nishihara et al., 2022;Nishiyama et al., 2020; Ochi et al., 2020;Ohara et al., 2021;Qin et al., 2021, 2020;Sahin et al., 2021;Shi et al., 2023;Sun et al., 2020;Tang et al., 2018, 2016; Terp et al., 2021;Tong et al., 2019;Wang et al., 2023; T.-H.Wang et al., 2020;Watanabe et al., 2021;Yu et al., 2020; L.Zhu et al., 2021)
Rociletinib	EGFR with T790 mutation	Irreversible		(Fawwaz et al., 2021, 2020; Han et al., 2020; Tang et al., 2016; Umeda et al., 2017)
TAS-121	EGFR with T790 and L858R mutation (exon 19 and exon 21)	*not specified*		(Watanabe et al., 2021)
Tyrphostin AG-1478 (NSC 693255)	ErbB2 and PDGFR	*not specified*		(Koizumi et al., 2005; Nakachi et al., 2010)
Vandetanib	VEGF2, VEGF3, EGFR, RET, and to a lesser extent VEGF1	*not specified*		(Ichihara et al., 2009)
WZ4002	EGFR (L858R and T790M), AKT and ERK1/2	*not specified*		(Ji et al., 2020; Raoof et al., 2019; Umeda et al., 2017)
ZD6474	VEGFR, EGFR and RET	Reversible ATP binding competitor		(Arao et al., 2004)
Zipalertinib (TAS6417)	pan-mutation-selective EGFR	ATP competitive inhibitor		(Hasako et al., 2018)

showing potential properties to be targeted have been found. It is about the anti-apoptotic B-cell lymphoma 2 (Bcl-2), markedly increased in resistant clones, and the Extracellular Signal Regulated Kinase (ERK) playing a role in drug-resistance, both potentially targeted to inhibit the Gefitinib resistance (Cheong et al., 2018; Ochi et al., 2014; Qi et al., 2018).

The identification of abnormal expression of molecules even involved in different pathways, can pave the way for the use of other inhibitors in combination with the existing EGFR-TKIs to improve the relative sensitivity of resistant cells. An example is that reported by Cai and colleagues which tested the combination approach of the antimalarial drug Dihydroartemisinin (DHA) with Osimertinib demonstrating the ability to reverse the resistance and the increase in ROS impairing the heme metabolism (Cai et al., 2021).

The choice to assess the mechanisms involved in the most recent Osimertinib resistance, due to the relatively recent approval, led many authors to test the role exerted by other off-target drugs, inhibitors or potential ones, on EGFR-mutant NSCLC. The investigation of CFM 4.17, a CARP-1 inhibitor, has been tested to be used as a pre-treatment to overcome Osimertinib resistance, since it occupies the active pocket of the EGFR active site, with the same aim tested the Abemaciclib, a dual CDK4/6 inhibitor used for the treatment of metastatic breast cancer, to treat TKI-resistant mutated NSCLC. The combination of Osimertinib with Abemaciclib, in fact, suggested a potential approach to prevent or delay Osimertinib resistance in first-line treatment (Kommineni et al., 2021; La Monica et al., 2020).

However, besides the resistance against Gefitinib, RPC-9b cells demonstrated sensitivity to 90 kDa heat shock protein (Hsp90) inhibitors. The Hsp90 inhibitor 17-DMAG (17-dimethylaminoethylamino-17-demethoxygeldanamycin) and Ganetespib, a second-generation HSP90 inhibitor with anti-cancer properties, was employed to assess the anti-proliferative effects against mutated and resistant cell line showing potential clinical efficacy (Kobayashi et al., 2012; Kurihara et al., 2019).

Several other inhibitors were also used, for example those against MET (Yu et al., 2020). These authors employed the MET inhibitor HQP8361 both as a single agent and in combination with Osimertinib in Osimertinib resistant cells. Since the increased levels of MET and p-Met in these latter with respect to the sensitive ones, the synergistic approach of these two different inhibitors results in a reduced survival of HCC827 Osimertinib resistant cells.

Since their mutational profile, PC9 cell line results one of the most employed cells as a valuable model to study the resistance induced by several TKIs. The Erlotinib resistant PC9 (PC9-ER) were employed to assess the Efatutazone, a novel peroxisome proliferator-activated receptor gamma (PPARγ) agonist, in the motility inhibition ability (Serizawa et al., 2014). Once again, the combination strategy using Efatutazone plus Erlotinib result in the inhibition of PC9-ER cell migration greater than that of Efatutazone or Erlotinib alone.

A particular finding was the role exerted by mTOR and the related pathways, which seems to be different between sensitive and resistant NSCLC cell lines (Fei et al., 2013). This time, Gefitinib resistant PC9, together with H1650 and H1975 cells, were employed to detect mTOR signaling pathways through western blot and immunoprecipitation assays, suggesting the relative inhibitors as a good strategy for patients with EGFR mutant NSCLC who develop EGFR TKI resistance.

Another potent inhibitor, not EGFR-TKI, is the inhibitor of G9a histone methyltransferase BIX01294 (BIX). Its anti-cancer properties are

ascribable to the reduction of EGFR level in EGFR-mutant NSCLC, where it demonstrated to induce apoptotic effect with respect to the wild-type ones (J. H. Kim et al., 2021). The achievement of alternative strategies against EGFR-TKIs resistant NSCLC led to pursue a combined inhibition of FGFR and Akt pathways by employing Erlotinib, Gefitinib, and Osimertinib EGFR-TKI-resistant cell line models, showing a synergistic strategy better than the combined approach EGFR-FGFR inhibition with an increased therapeutic efficacy (Terp et al., 2021).

3.5.3. RNA-mediated strategies

The use of RNA molecules, involved in gene regulation and silencing, constitutes a frequent strategy followed by several authors, often coupled with other approaches. The small interfering RNA (siRNA), the short-hairpin RNA (shRNA), the micro RNA (miRNA), and the circular ones (circRNA), represent a valid tools to overcome the resistance induced by TKIs. The control of the expression of specific genes by knock-down regulation and silencing processes can lead to enhance the efficacy of the treatment or simply to demonstrate the involvement of some molecules and pathways in the anti-cancer activity of the drug employed.

The involvement of BIM (BCL2-like 11) in the apoptosis induced by Gefitinib in EGFR mutant cells (H1650, HCC827, H3255 and PC9), was demonstrated by Costa and colleagues through the employment of a siRNA (Costa et al., 2007). The knockdown of BIM, in fact, demonstrated to attenuate the apoptosis in Gefitinib treated cells. Li and colleagues, (Y.-Y. Li et al., 2013) focusing on the autophagy as protective mechanism against Erlotinib resistance, and hence enhancing NSCLC survival. To do so, they tested four different cell lines with different mutational status, by performing a knockdown mediated by siRNA against Atg5 and Beclin 1, involved in the mechanism. The inhibition of the autophagy produced an improved sensitivity to Erlotinib, highlighting its involvement in the resistance mechanism.

How the combinational approach between different drugs can be valuable has been demonstrated in the previous paragraph, but sometimes the additional efficacy of a siRNA mediated knockdown can be more. Several cell lines have been chosen for the different expression of LKB1, an antiproliferative mediator of metformin, to study its involvement in the combined approach Metformin-Gefitinib in NSCLC cells (Morgillo et al., 2013). About Icotinib, instead, PC9 and HCC827 cells were used to evaluate the knockdown effects of Casitas-B-lineage lymphoma protein-B (Cbl-b), an E3 ubiquitin ligase, to clarify its role in the resistance against this first generation TKI (T. Zhang et al., 2019). In order to identify the potential causes of TKIs resistance, a PC9/AB2 cell line was established with 576-fold decrease in gefitinib sensitivity compared with its parental PC9 cell lines (Ju et al., 2010). PC9/AB2 demonstrated increased adhesion and migratory properties associated with an increase of Integrin-β1 expression. A negative correlation between the sensitivity of NSCLC cells to Gefitinib and its expression rate was found, thus offering an important role in Gefitinib resistance.

Even among the silencing strategies, the employment of the pLKO.1 lentiviral vectors allowed the expression of the shRNA against PTEN (Kim et al., 2013). The downregulation of PTEN in Gefitinib resistant cell lines to induce radioresistance, was combined with the inhibition of mTOR mediated by the relative siRNA, in order to radiosensitize NSCLC harboring the EGFR activating mutation. These results demonstrated that acquires resistance to both TKIs and radiotherapy is due to PTEN loss or inactivation mutations.

The role played by the Adenine Nucleotide Translocase-2 (ANT2), an oncogenic mitochondrial protein, seems to be involved in Gefitinib resistance, since its high expression in Gefitinib resistant (H1975 and HCC827-GR) with respect to the Gefitinib sensitive cell lines (HCC827and PC9) (Jang et al., 2016). Its high expression nominates ANT2 to be an excellent resistance biomarker; therefore, the inhibition of ANT2 could represent an alternative strategy for overcoming the resistance against Gefitinib treatment. Remaining in Gefitinib resistant contest, another biomarker can be represented by EI24 a tumor suppressor encoding gene, highly present in p53 expressing cells. Choi and colleagues employed the PC9-GR cells to deepen the role exerted by EI24, and demonstrating how its knockdown lead to restore the sensitivity to Gefitinib (Choi et al., 2015).

Another well-assessed RNA-based strategy pursued, is that concerning miRNAs. Their involvement in cancer development, constitute an important marker to study. These small endogenous RNAs constitute a potential target to attenuate resistance to TKIs, since its implication in the initiation and progression in many forms of cancers. Gefitinib-resistant PC9 (PC9-GR) were used to overcome the resistance by studying both miR-138–5p and miR-21 generally involved in resistance against cytotoxic agents (Gao et al., 2014; Li et al., 2014). miR133a-3p, instead, was targeted to induce an its own overexpression to increase the Gefitinib sensitivity, since its down-regulation in Gefitinib resistant cells (Q. Li et al., 2021). Positive effects were also observed in the over-expression of miR200-a, which is normally down-regulated in NSCLC cells, while its enhancement demonstrated good ability in the inhibition of cell migration and invasion (Zhen et al., 2015). On the other side, the overexpression of miR-21 seems involved in the resistance against cytotoxic agents. Once again, the peculiar profile of PC9 cells lends itself to study the association between its overexpression and the resistance against TKIs (Li et al., 2014). H1650, H460 and H1975 demonstrated valuable models to overcome the Gefitinib resistance testing the combination therapy with miR-30a-5p. The achieved results highlighted the innovative plan, acting through the PI3K/AKT pathway (F. Wang et al., 2020).

As well as for miRNAs, also circular RNAs are related to the acquired Gefitinib resistance. Their not well-known role is still debated, and further insights are still analyzed. Chen and colleagues (T. Chen et al., 2019), for example, employed Osimertinib-resistant H1975 and HCC827 cell lines to deepen the role exerted by circular RNAs and the role they played in cancers, whereas Wen and colleagues (Wen et al., 2020) evaluated the expression and functional profile of circRNAs in resistant rather than sensitive PC9 and HCC827. What emerged by these studies is their role in the Osimertinib and Gefitinib resistance, respectively. In the first case, H1975, HCC827 and Osimertinib resistant H1975 (H1975/AZDR) revealed a lot of circRNAs with different expression rate, among which several of them involved in Osimertinib associated resistance, thus, to be valid candidates as biomarkers. On the other hand, the screening performed by Wen and colleagues showed the differences found between Gefitinib sensitive and resistant NSCLC cells, making a comprehensive expression and functional profiles of the differentially expressed circRNAs.

3.5.4. Approaches to overcome the Epithelial Mesenchymal Transition (EMT)

The EMT is one of the possible phenotype transformation induced by the TKI resistance mechanisms related to invasion and metastasis of tumors. It consists of the transition to a mesenchymal phenotype from an epithelial one, leading to a change in cell polarity and adhesion. To study the related issues, TKI-resistant cell lines must be established in order to observe mesenchymal properties, and in most cases the studies reported deal with strategies previously described.

Some researchers, for example, established an acquired Gefitinib-resistant cell line, which exhibited EMT and stem cell-like properties to study the overcoming of resistance by silencing the expression of tumor necrosis factor receptor superfamily member 19 (TNFRSF19), also known as TROY (Wu et al., 2022).

The same resistant cell line was used to evaluate the increase of MET gene copy number. This gene amplification allows proliferation, resistance and migration enhancing the EMT, leading to hypothesize a possible targeted therapy or preventing strategies in those patients with advanced or metastatic NSCLC with acquired EGFR-TKI resistance in which there is a MET gene amplification (Baldacci et al., 2018; Cappuzzo et al., 2009). Since its involvement in the EMT context, a possible target could be represented by Snail, a related transcription factor

regulating the E-cadherin expression. In this case, the CDK4/6 inhibitor Palbociclib was used to test the ability of reversing drug resistance by downregulating Snail (Qin et al., 2021). Its up regulation in Osimertinib resistant H1975 lead it to be targetable to control the resistance against third generation TKI. Moreover, unusual T790M mutated cell lines have been used in the epithelial mesenchymal transition (EMT) context, where the authors focused on the FGFR together with EGFR, suggesting a promising synergistic target to prevent the EMT acquired resistance to TKIs in EGFR mutated NSCLC (Raoof et al., 2019).

In a pandemic situation like the one experienced in 2020–2022 was not excluded the employment of Gefitinib- and Erlotinib-resistant cell lines as suitable models to study the Sars-CoV2 pathogenesis and that related to other respiratory viruses (Stewart et al., 2021). The relation between this aim and the Epithelial Mesenchymal Transition lies in the metabolic and transcriptional changes occurring in epithelial cells during the infection.

Coming back to miRNA-based approaches, their role was not excluded from this context. In fact, it was demonstrated that the chronic treatment with Gefitinib induced a reduction in the expression of miR-155 and miR-200c and their depletion has been associated with EMT and hence with the gaining of Gefitinib resistance (Narita et al., 2017). Among all the issues, the less investigated in this context was just the EMT, except in more recent articles, when PC9 and HCC827 cell lines were used as a representative phenotype without considering other genetic arrangements. Being one of the most frequent consequences of acquired resistance to TKIs, the EMT is a phenomenon that can be reverted targeting the molecules promoting the transition (A.-F. Lee et al., 2017). Moreover, the expression profile after the EMT induction mediated by TGF-β, demonstrating the involvement of miR-134/miR-487b/miR-655 in reversing the transition and in enhancing TKI resistance, suggesting these ones can be new targets in advanced lung adenocarcinoma (Kitamura et al., 2014).

Another target of the EMT is represented by miR-19a, a regulating cMet miRNA, that is down-regulated in Gefitinib-resistant cells promoting the mesenchymal phenotype, and can be used as a valuable biomarker detectable at the serum level (Cao et al., 2017). Its down-regulation, in fact, suppress the expression of epithelial markers by promoting the expression of mesenchymal ones.

3.5.5. *Antibodies mediated strategies*

Monoclonal, nano- or micro-, Antibodies (Ab) are widely used in several strategies and with different approaches. In this context, only a few works reported about the combinatorial strategy using these biological tools, even with different aims.

Among the monoclonal antibodies, Onartuzumab, an anti-MET mAb, and Cetuximab, an anti-EGFR mAb, were employed. Differently for other strategies seen till now, the employment of the monoclonal antibody Cetuximab was used to test the possible radiosensitization of different NSCLC cells both in vivo and in vitro (Yonesaka et al., 2008). Conversely, the combination of Onartuzumab with Erlotinib was tested aiming to improve the therapeutic efficacy of this latter, by blocking the ligand of the MET receptor tyrosine kinase, the hepatocyte growth factor HGF, and inducing MET activation in PC9 cells (Sano et al., 2015). This fact lays the foundations for a possible treatment strategy for NSCLC with activating mutations and with a high expression of HGF. A different employment is that used to radiolabel the Onartuzumab (89Zr-onartuzumab PET) to monitor c-Met status with the aim to monitor drug efficacy or emergence of resistance in HCC827 Erlotinib resistant cells (Pool et al., 2017). The radiolabeling, allowing to discriminate changes about c-Met expression, makes it a valuable resistance-related biomarker.

Another innovative strategy employed to overcome the resistance is to target the Epidermal Growth Factor (EGF) blocking the first phases of EGFR pathway. To do so, the anti-EGF nanobodies (Nbs) were employed with all the advantages offered instead of traditional mAbs, among which the ability to target the soluble tumor growth factors (Guardiola et al., 2022). In this case, the combination of Osimertinib with Nbs in PC-9 resistant cells enhances the efficacy of the TKI favoring apoptosis and reducing cancer stem cell-like properties able to promote metastasis.

3.5.6. *Dietary and natural compounds based strategies*

Since more than a decade, dietary compounds represent a valid and widely studied anti-cancer option in addition to other well-known inhibitors and drugs. The impact of natural compounds continues to gain the attention due to their potential and synergistic properties in combination with several TKIs, showing anti-apoptotic or inhibitory effects. The unique example of cell lines both harboring T790M and C797S mutations is reported by Shi and colleagues (Shi et al., 2023) by using the mutated mouse Lewis Lung Carcinoma cell line (LLC). These cells were obtained by a lentivirus-mediated transfection and employed in this context to test Osimertinib and the clinical Chinese formula "Feiyiliu Mixture" to enhance and then demonstrate the activation of anti-proliferative and anti-apoptotic pathways, as well as the efficacy and the reduced toxicity of the treatment.

Remaining in the Chinese medicine tradition, the Gambogic acid (GA) extracted by the Guttiferae plant "Garcinia hanburyian", is an alternative medicine used in the treatment of several diseases (Wang et al., 2015). The synergistic effect of GA with Gefitinib showed promising results in H1975 tumor growth in vivo, evidencing the apoptotic effect and suppressing the gefitinib-resistant tumor growth. Some researchers (Z. Chen et al., 2022) used another known Chinese product, berberine, acting as a MET inhibitor that seems to overcome Osimertinib resistance amplifying its effects. The combined approach of Osimertinib was demonstrated to be a valid strategy also in combination with Anlotinib, a VEGFR-TKI acting synergistically inactivating c-MET/MYC/AXL axis (Lei et al., 2023). Even its usage in the treatment of diabetes in ancient China, the Huanglian Jiedu Decoction (HJD), with its liver detoxification and protective properties, has been employed in HCC827 and H1975 cell lines to promote the Erlotinib induced apoptosis and the inhibition of Bcl-XL, Bcl-2, and p-STAT3 expression (Zhou et al., 2021).

On the other hand, the combined treatment of Erlotinib with natural derived products was a frequent strategy adopted by several authors to exploit their wondrous effects in EGFR-mutant NSCLC, for example by using the dietary flavonoid ampelopsin and the root extract of Peucedanum praeruptorum Dunn (EPP) (Hong et al., 2017; Park et al., 2022). The therapeutic effects exerted by curcumin against cancer are well-assessed, and the combination with Gefitinib in H1975 was demonstrated to enhance the anti-cancer effect of Gefitinib in resistant cells, comparably to the highest doses of the TKI used alone, also blocking the EGFR activation (Lee et al., 2011). Another natural active ingredient found in pepper plant, is the capsaicin able to enhance the citotoxicity of Erlotinib through the AKT inactivation mediated by ERCC1 down-regulation (J.-C. Chen et al., 2019). Fisetin, instead, a dietary phytochemical found in many fruits and vegetables, revealed to be able to reverse the resistance induced by Erlotinib in HCC827 cell line. This compound can inhibit the cell viability and hence inducing apoptosis through the inactivation of AXL, MAPK and AKT pathways and repressing Survivin expression (Zhang et al., 2016). Also pigments demonstrated apoptotic effects on lung cancer cells, as those reported by the green pigment extracted from Calophyllum inophyllum L. against H1975 cell line (Hsieh et al., 2018). Trifolium flavonoids (TF) properties were established employing PC9 Gefitinib resistant cells. The combination of TF with Gefitinib induced a highest apoptotic rate with respect to the single administration of the TKI, highlighting once again the potentiality of the synergistic combination of a natural compounds with a commonly used TKI (Wu et al., 2020).

4. Discussion

The importance to establish an experimental model, that is as close as possible to the clinical question, represents a continuous challenge to better evaluate in vitro what really happens in vivo. Nowadays, the

scientific community has exemplary laboratory models that allow to study and deepen in vitro some clinical issues, from cell-based systems to animal and patients-derived models (Hou et al., 2022). In the oncological scenario, it becomes even more important to employ specific and ad-hoc systems that can mimic the environmental conditions of a tumor. In this way, it is possible to reproduce in the laboratory what really happens in vivo, allowing to test and then to setup what can be really effective in humans (Martinez-Pacheco and O'Driscoll, 2021; Özdemir et al., 2023). Nowadays, the cell lines continue to represent the most handling and easy to use in vitro model, quite always employed as an alternative to animal tests or together with animal or clinical models, respectively for the in vivo and ex vivo studies, despite the more realistic 3D cell-based spheroids and organoids. These latter 3D models mimic the spatial contribution of solid tumors even if with significant differences between them. Anyway, spheroids and organoids constitute a valuable comparative pre-clinical model able to assess drug sensitivity, and to predict treatment response and hence the occurrence of resistance (Bie et al., 2021; J.-H. Chen et al., 2020; Liu et al., 2020; Theard et al., 2020). On the other hand, cell lines constitute a shared and fair experimental model that excludes too many variables due to the intra- and inter-individual variability occurring in clinical and animal models. The choice to operate with immortalized cells is the preferred way in almost all cases with respect to the primary cell cultures. This is mainly due to the senescence that primary cells encounter, which over time tend to change and stop replicating. From a phenotypical point of view, the culture of the primary pulmonary Human Small Airway Epithelial Cells HSAEC (Fig. 4a), the immortalized NSCLC wild-type A549 cell line (Fig. 4b) and the mutated NSCLC cell lines PC9 (Fig. 4c) appear similar, but the advantage to use immortalized cells lies in avoiding early senescence and in a more lasting model.

However, despite these advantages, cell lines can be affected by genetic rearrangements because of the divisions they are subjected to, that together with different manipulation protocols by different scientists may create possible differences in terms of results obtained.

In this review the attention has been paid to one of the main worldwide oncological burden represented by lung cancer, in particular on the most represented, NSCLC, mainly focusing on the so-called oncogene-addicted cancers. In fact, 15–20% of the total NSCLCs are characterized by specific mutations affecting the tyrosine kinase EGFR. These mutations can be classified as sensitivity mutations, when they appear and can be targetable by a specific TKI based treatment, acquired resistance mutations or also defined secondary mutations, when they develop as a consequence of the therapy itself.

For the first time to the authors knowledge, this review wants to retrace an overview of existing literature about EGFR mutated cells. In particular on tumor immortalized cells obtained from different NSCLC histotypes, up to date used to study the development of resistance after TKIs treatments, by mentioning the most peculiar works found in literature. The aim was to assess the profiles of the most employed NSCLC mutated cell lines and the strategies pursued to overcome the resistance against EGFR-TKIs used in clinical practice. In this way, we pointed out the current status about this topic, by highlighting the best representative EGFR mutated cell models employed to evaluate possible alternative treatments that could be tested in the clinical reality.

Even though all the articles, which were selected over a period of time of 24 years, met the same eligibility criteria, the experimental strategies performed were largely different. After the screening, 228 papers were selected providing for original articles dealing with at least one EGFR mutated NSCLC and at least one EGFR-TKI treatment. What emerged, in addition to the cell line models and to the TKIs treatment of interest, the selected articles revealed many other experimental models, studied together with other tumor cell lines and not EGFR mutated NSCLC cell lines, human specimens and mice models to perform in vivo and ex vivo analyses. About cell line models, many authors used several of them from wild-type NSCLC to NSCLC mutated ones but with other genetic arrangements not affecting EGFR, including further tumor epithelial cell lines arising from other malignancies, such as prostate, breast mammary gland, ovary, kidney, gastric site or simply other lung cancers. Once respected the established criteria, papers have been evaluated and analyzed according to the approach investigated.

Through this investigation a critical consideration has emerged. In fact, basic and specific information about the mutational profile of cell lines used is not always clearly reported, and in some papers cannot match with what reported in the corresponding purchase site. Missing or incomplete data are also those related to the TKI concentrations employed. In most cases the authors reported a range of TKIs concentration without specifying the exact doses used, and what strikes even more is to find dissimilar values for the same cell lines and the same treatment among different works. This means there is a need to be consistent and scientifically accurate to really help and improve not only the own work but also the international research community. In this way, it will be possible to certainly enhance the scientific works through detailed and more reproducible descriptions, useful for those who will approach similar research. In this way, it will be possible to define a specific in vitro model, for example for a specific mutational profile, thus to investigate and deepen some issues related to the resistance mechanisms occurring in a patient affected by the same tumor phenotype harboring the same mutation.

Otherwise, for the most the in vitro experiments reported reflect the clinical reality in terms of mutated cell models and issues faced. In fact, not surprisingly, given the highest presence of EGFR mutations, adenocarcinoma stands as the most considered NSCLC mutated histotype, and among the TKIs known and clinically applied to date, Gefitinib, Erlotinib and Osimertinib as the most employed and studied EGFR

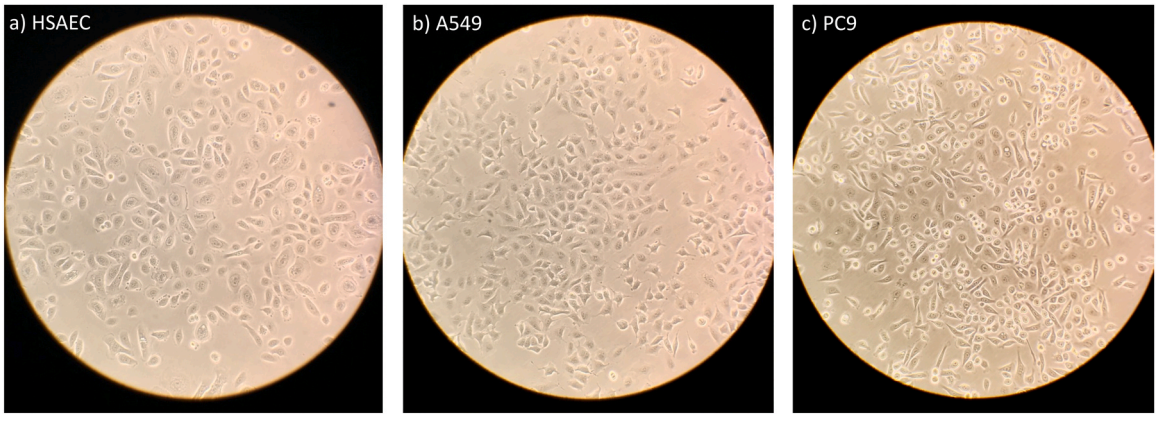

Fig. 4. 20x magnification representative images of the (a) primary Human Small Airway Epithelial Cells HSAEC, (b) A549 and (c) PC9 cell lines from human lung adenocarcinoma.

mutated inhibitors.

Regardless cell line models and EGFR-TKIs approached, the purpose of the research is common: to find a strategy to overcome the resistance developed after TKI treatment. Whether it is a well-known inhibitor or potentially dietary product, rather than an RNA or Ab-mediated technology, the common aim is to avoid the resistance and all the secondary effects produced by combining them or other well-known off-target inhibitors with the TKI of interest, often in a synergistic way. Targeting specific pathways seems to be the most popular strategy even by using other TKIs, enhancing the inhibitory effects and the anti-cancer properties. RNA- and Ab-mediated technologies offer a great and suitable potential, as well as the employment of natural and dietary products, that showed good inhibitory activity. These interesting results should all match the clinical applicability, otherwise they would remain an end in themselves without really improving and solving current issues related to the occurring EGFR-TKIs resistance mechanisms in oncogene-addicted NSCLC.

5. Conclusions

The present scoping review aimed to frame the current advancement about oncogene-addicted NSCLC and the EGFR TKIs up to now used, focusing on the relative mutated cell line models and on strategies used to study the related resistance mechanisms. The analysis of all the original articles selected, found in literature from January 1999 to March 2023, allowed us to obtain the current perspective about the experimental in vitro models employed to study this phenomenon. The onset of resistance mutations let to employ mutated models in order to assess the consequences induced by the TKI administration and to study novel strategies to overcome the resistance. Together with the most known models and TKIs, several other cell lines emerged as valid models contributing to broaden the variability of studied NSCLC subtypes.

The importance to reverse the onset of TKI resistance, and hence to find a way to avoid that genetic arrangements occur, constitutes a priority within the oncogene-addicted NSCLC. Establishing a realistic model as close as possible to the clinical issue helps to improve the therapeutic strategy to be used in EGFR-mutated lung cancers. For these reasons, this review wants to resume the research performed about this topic over the years, by offering a selection of strategies pursued till now thus to assess the current situation and to proceed improving the scientific advancement in this topic using real strategies to approach clinical reality.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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