ISSN 2959-409X

Mechanisms of NSCLC EGFR-mutant Cell Lines Developing Treatment-Adaptive Resistance In vitro

Jingyi Zhu[1, 2, *]

[1] School of Biological Science, the University of Adelaide, Adelaide, Australia
[2]Haide Collage, Ocean University of China, Qingdao, Shandong, China
[*]Corresponding author: jingyi.zhu02@student.adelaide.edu.au

Abstract:

NSCLC ranks at the top of lung carcinoma cases worldwide, with three generations of EGFR-TKIs used in clinics where resistance derives ultimately. Molecular mechanisms have been underscored for their contribution to resistance. More recent studies reported the first-line therapy Osimertinib gives rise to the acquired T790M and C797S mutations, addressing the necessity of inhibitor usage. Therapeutic windows occur by the lung-specific hydroxylase mimicry and pVHL agonists development for HIF-1α high-accuracy degradation, considering the correlation between HIF-1α and other signaling pathways. This paper analyzed the in vitro research on the role of MAPK and PI3K-Akt pathway, NF-κB pathway, and hypoxia-regulated HIF-1α signaling in generating EGFR-TKI resistance and obtained the results of further validation in the inhibitors used for the pathways blockage. The pathways involved trigger the hallmarks of cancer and stimulate inflammation under stressed conditions. Meanwhile, inhibitors failed in their function due to misleading signal engagement, technical weakness, and off-target effects. With new target emergence, it drives a potential of constant disruption in resistance-associated pathways. However, the target feasibility and practical drug development pipeline have not been revealed and require future studies.

Keywords: EGFR-TKI resistance; non-small cell lung cancer; HIF-1α

1. Introduction

Lung carcinoma has always been a primary reason for universal cancer-associated death, where the most frequent subtype goes to non-small cell lung cancer (NSCLC) [1]. Instead of applying chemotherapy for advanced NSCLC patients, personalized treatments like tyrosine kinase inhibitors (TKIs) are highly recommended in clinics for the development of mutations in epidermal growth factor receptors (EGFR).

Commonly used EGFR-TKIs have been developed in three generations to inhibit EGFR downstream pathways implicating proliferation, invasion, and metastasis [1]. The first generation of TKIs is reversible ATP-competitive inhibitors for EGFR, including Gefitinib, Erlotinib, and Icotinib. Acquired resistance is developed within 9 to 14 months for the patients with EGFR mutants. Second-generation TKIs harbor the tyrosine kinase domain irreversibly, like Afatinib and Dacomitinib, to prevent the problems and result in improved survival but high adverse incidence. The third generation of TKIs is developed to address the issues, binding irreversibly to the cytosine in the ATP-binding site for EGFR tyrosine kinase domain, such as Osimertinib. Different TKIs are used respectfully for patients, and all of them are clinically relevant.

Despite that, the disease relapse with drug resistance is observed in patients with third-generation EGFR-TKI treatment, although it is predicted to act as the resistance-free version of TKIs in the first place. However, the essential pathways helping cells develop resistance are still uncovered, including the signaling pathway regulating the enrichment of cancer hallmarks and the promoted stress for acquired sub-mutations. The hypothesis of MAPK and PI3K-Akt pathway, NF-κB pathway, and hypoxia-regulated HIF-1α signaling made up the majority of research interests. Acquired mutations like EGFR T790M are frequently observed in the resistant cells, which indicates another potential driving force [2]. These are closely related to cell cycle progression, cell proliferation, invasion, and anti-apoptosis, making progress for the NSCLC. What types of secondary mutations might give rise to drug resistance can signpost the direction of drug discovery. However, focusing on the signaling pathways may produce more probability of containing broader annotations to prevent resistance derivation. Recent research addressed some inhibitors for the signaling pathways, like Dactolisib for MAPK-Akt pathways, which could conquer

the EGFR-TKI resistance but still needs optimization and further research.

This essay analyzed different pathways involved in EGFR-TKI resistance in vitro to overview their contribution to eliminating TKI resistance. Moreover, it also provided the optimization for an outlook on the combination of drugs or targeted sites blocking the pathways for resistance in future studies.

2. Mechanisms of Treatment-Adaptive Resistance

Treatment-adaptive resistance, or EGFR-TKI resistance, describes the resistance derived after incubating with EGFR-TKI in cell culturing. The experiments aimed to reduce the resistant effect will generate the resistant cell lines prioritize any other drug incorporation, but the exact mechanism driving the resistance occurrence remains unclear. The MAPK-AKT signaling, NF-κB signaling, and HIF signaling are the hot-spot pathways for research conduction. With more and more discoveries in secondary mutation T790M observed continuously crossing the subjects, it is also considered a driving force of resistance.

2.1 MAPK-AKT Signalling Pathway

The mitogen-activated protein kinase (MAPK)-protein kinase B (AKT) signaling pathway describes the crosstalk between the two pathways to be triggered by the EGFR ligand binding. The over-activated EGFR leads to the over-phosphorylated methyl ethyl ketone and Akt, resulting in activated MAPK that is inhibited by phosphorylated Akt to a certain extent and inhibited tuberous sclerosis complex 2 [3]. Consequently, the mammalian target of rapamycin complex 1 (mTORC1) is hyperactivated for enhanced cell proliferation, migration, and survival. mTORC1 also blocks the binding of eIF4E-binding protein to the elF4E for mRNA over-transcription, which could cause tumors in mice models. The hyperactivation of the pathways is correlated with the further development of disease with more intensive cancer hallmarks incorporation, leading to a severe outcome for the patients.

The MAPK signaling-induced EGFR-TKI resistance was observed by Eberlein‘s team in 2015 [4], addressing the independent activation of the MAPK pathway from EGFR could give rise to TKI resistance. The methyl ethyl ketone- the precursor of MAPK- inhibition, which is observed to be more sensitive in the resistance cells, links the origin of MAPK over-activity with resistance occurrence. Furthermore, the EGFR-TKI resistance is regulated by AKT signaling, which is proved by Wang’s experiment in 2018 [5]. The phosphorylated AKT could self-activate the EGFR-TKI resistance in the exon 19 deletion cells, with the association of downregulated Bcl-2 to inhibit the occurrence of apoptosis within the tumor cells.

Moreover, the combination of MAPK-AKT signaling reactivation is further detected for continuous resistance of EGFR-TKI Osimertinib [3]. These two pathways are reactivated after treating the TKI, and the inhibition of the cell growth effect is attenuated other than initially promoted by another agent. The MAPK-AKT pathways can be monitored via the overproduction of MAPK and the higher expression of mTORC1 or by comparing the phosphorylated methyl ethyl ketone and AKT between the resistant cell lines with the non-resistant version of cells.

Consequently, several researches have proved that the MAPK-AKT pathway could be responsible for the EGFR-TKI resistance, which can be easily measured by testing the difference in the expression level between the resistant and normal NSCLC cell lines.

When receiving the paper, we assume that the corresponding authors grant us the copyright to use the paper for the book or journal in question.

When receiving the paper, we assume that the corresponding authors grant us the copyright to use the paper for the book or journal in question. When receiving the paper, we assume that the corresponding authors grant us the copyright to use.

2.2 NF-B Signalling Pathway

Nuclear factor kappa B (NF-κB) is a transcription factor composed of p65 and p50. The EGF receptor regulates it and becomes active when inhibitory proteins like p105 and IκBα are processed or degraded, causing the subsequent cell proliferation, apoptosis, and inflammation to be triggered. The overactivation of the EGFR leads to the overproduced NF-κB, which supports cancerous feature enhancement.

The correlation between NF-κB and tyrosine kinase inhibitors was demonstrated earlier in 2014 by Seguin’s team [6]. It suggested that the NF-κB activation leads to the promoted receptor tyrosine kinase inhibitor resistance in the lung carcinoma cell line. Despite that, they also signposted several types of machinery that could have NF-κB involved, such as anchorage independence, self-renewal, and epithelial-to-mesenchymal transition, vague to suggest the determinant role in promoting TKI resistance.

Afterward, Blakely‘s research unveiled the survival under EGFR TKI treatment, modulated by NF-κB signaling [7]. They validate the cleavage of inhibitory proteins within the NF-κB activating complex due to EGFR oncogene inhibition specifically for the TKI therapy, which is inspired by the clinical response. By further suppressing the NF-κB pathway, they observed decreased resistance emergence. However, the NF-κB inhibitor PBS-1086 performed a better outcome with the combination of EGFR TKI for resistance-free survival at the beginning, then dropped gradually after a couple more days of cultivation,

which could be limited due to the selection of the drugs. But otherwise, the results informed the role of NF-κB signaling in EGFR-TKI resistance.

In addition, NF-κB becomes further activated by the induction of the AKT signaling pathway, as mentioned above [8]. The Akt is responsible for the phosphorylation of inhibitory proteins, leading to the degradation and release of NF-κB into the nucleus to regulate the cellular response affecting cancer enhancement. This could act as another pharmaceutical target to interrupt NF-κB signaling.

To summarise, EGFR dysregulation leads to the hyperactivated NF-κB signaling, mediating the cell proliferation and inflammation that triggers the EGFR-TKI resistance. It could be the therapeutic target to overcome the resistance, which is produced to avoid the decrease presented by the early version of the NF-κB inhibitors and hopefully extend the effect duration.

2.3 HIF-1α and HIF Signalling Pathway

Although the tumor microenvironment is hard to investigate in the in vitro experiment, the hypoxia-inducible factor 1 alpha (HIF-1α) provoked by the TKI-triggered cellular stress is sufficient to induce drug resistance through the EGFR signaling activation [9]. The HIF can be activated by the hydroxylases that sense oxygen and hydroxylase the HIF-1α under the normoxia condition. With the phosphorylated von-Hippel Lindau (pVHL) attachments, HIF-1α is linked by the polyubiquitin chain for degradation. When the tumor microenvironment is hypoxic, hydroxylases cannot add the hydroxyl group to the HIF-1α; it is stabilized by the binding of CREB binding protein/p300 to the N-terminus. By the pairing of HIF-1β, it transcribes genes that contribute to glucose consumption and angiogenesis, supporting the nutrition and invasion of the lung tumors.

HIF-1α has been shown to up-regulate proliferation through EGFR signaling in some NSCLC cell lines [10]. It was observed by Meng's team early in 2007 but demonstrated new properties in their recent studies, relating the positive regulation between HIF-1α and c-Jun to derive acquired resistance. They were inspired by the increased expression of HIF-1α protein in the acquired resistant NSCLC cells with EGFR mutation. They then dug into the correlation of HIF-1α with c-Jun regulation under hypoxia conditions. The positive feed-forward loop presented sufficiently illustrates their contribution to resistance development by promoting the downstream signaling of the EGFR pathways. Regardless of using the first-generation EGFR-TKI, the frequent resistance in drug usage still indicated the potential therapeutic targets of their findings.

Recent studies focused on the inhibitor of HIF-1α, the YC-1, to suppress tumor growth and angiogenesis in both normoxia and hypoxia environments [11]. It abolished the HIF-1α production, as single gefitinib therapy promoted its protein level but was reduced by the combined treatment. It also involved kinase-independent signaling for the degradation of EGFR, which eliminated the downstream signaling set off by the EGFR mutation. The building of gefitinib-resistant NSCLC cell lines was treated by YC-1, resulting in induced intrinsic apoptosis with decreased EGFR protein as its anticancer effect. These findings suggested a promising future for investigating the potential targets of HIF-1α signaling for resistance overcoming in multiple generations of EGFR-TKIs.

To summarise, HIF-1α and HIF-signalling stimulate tumor growth and angiogenesis in EGFR-TKI pre-treated cells, mimicking the stressed condition in vitro and addressing the prospective target to conquer resistance.

2.4 Secondary Mutation T790M

The early generation of EGFR-TKIs used in clinics has been reported to acquire resistance by the secondary mutation EGFR T790M [12]. It is widely reduced by the Osimertinib engagement, which aims to lock the ATP-binding pocket and prevent drug resistance aroused by T790M. However, pre-induced Osimertinib surrendered to prolonged treatment and gave rise to a secondary mutation T790M in NSCLC cell lines [13]. Although applying other TKIs could remove the concerns, this indicates that the efficient target to stop the secondary T790M, which eventually evokes resistance, remains indistinct.

In conclusion, the MAPK-AKT signaling, NF-κB signaling, and HIF signaling, with the secondary EGFR T790M mutation, observed to be up-regulated in the drug-resistant cells and being predicted to be responsible for the TKI resistance. They triggered the enhanced cell growth, proliferation, anti-apoptosis effect, invasion, inflammation for the stressed condition in the tumor microenvironment, and angiogenesis. Any mechanisms described above provide sufficient angles toward the development of new targets in the future.

3. Prospect Evaluation of the Findings

3.1 Drugs Used in Clinics

Although the third generation of EGFR-TKIs is designed to prevent drug resistance, it is still inevitably driving the cells for adaptation. According to the AURA trial consequences [14], some patients with untreated EGFR mutation using Osimertinib therapy emerged with secondary mutation for resistance, indicating that acquired resistance can be obtained in patients with enhanced clinical outcomes drug. This has also been proved by in vitro experiments. Numerous research groups can use low doses of Osimertinib to treat the NSCLC cell lines and acquire the

resistant cell line for further validation. For instance, Ichihara's lab [15] generated the resistance cells by incorporating different dosages of Osimertinib and refreshing the media during cultivation. The introduction of several cell lines with separate mutations prevents the occurrence of resistance bias to a single mutation.

Consequently, inhibitors for those downstream signaling pathways and secondary mutations are released for recurrence reduction and disease relapse prevention.

One of the PI3K-Akt inhibitors, Dactolisib, applied with Osimertinib, could effectively overcome resistance by Zhang's team [16]. They mitigated PTPN, a phosphatase regulated by EGFR, which could promote the MAPK pathway but inhibit PI3K signaling. The Akt hypophosphorylation can be achieved by the Dactolisib combination for resistance remission. However, considering the co-regulation of the MAPK pathway and PI3K-Akt, resistance reduction can easily be adverse to other regulatory machinery.

RNAi technique can be used for the inhibition of NF-κB [17]. This presents the potential for a therapeutic target but could be reversed by IRAK1 expression, where the microRNAs bind to the 3'-UTR of IRAK1 that initially causes NF-κB inhibitory protein degradation, ceasing the NF-κB from signaling. Another application of RNAi is involved in HIF-1α downregulation [18], where small interference RNA of Yes-associated protein alleviates hypoxia and induces apoptosis for TKI-resistance suppression. The limitations occur from the insufficient delivery efficacy, transient expression for repeated dosage, and non-accurate silencing for side effects of the RNAi technology itself.

Antibody remains a beneficial tool with Osimertinib, introducing the Bevacizumab for TKI re-sensitising triggered by acquired T790M mutation [19], unexpectedly no different in progression-free survival. Treating first- or second-generation TKI with acquired mutation by Osimertinib engagement is another recommended option [20].

3.2 Require Optimisation

Considering that third-generation TKIs have failed to stop the pathways that might contribute to resistance, with more recently reported patients having acquired C797S mutation, the urgent need for a next-generation TKI is apparent. A fourth-generation TKI was developed: EAI045, an allosteric inhibitor that has been developed to overcome the steric hindrance posed by the residues within the kinase domain [21]. With covalent binding and high selectivity to inhibit both acquired T790M and C797S mutations, it cannot act individually due to receptor dimerization.

Therefore, optimization is still required in response to better clinical outcomes.

3.3 New Target Development

As the crosstalk between HIF-1 and the signalling pathways mentioned above was revealed by Tang's team in 2023 [22], the HIF-1α protein reduction owns a higher potency to be a therapeutic target. The article suggested that HIF-1α can be induced by PI3K-Akt and MAPK signaling pathways, where NF-κB could suppress the oxygen detector hydroxylases and accumulate those HIF-1α. In this case, a HIF-1α inhibitor like YC-1 [11] and PX478 [23], combined with a pVHL mimic or agonist, could mitigate EGFR-TKI resistance. Both inhibitors mentioned above could regulate the HIF-1 protein level by inducing the hydroxylases for HIF-1α degradation; a pVHL mimic or agonist could act specifically inside the NSCLC cells for mimicry of its natural degradation process but become advanced. Accordingly, a specific hydroxylase agonist for HIF-1α regulation in NSCLC could also produce a therapeutic window for exploration.

4. Conclusion

In conclusion, various mechanisms could drive the EGFR-TKI resistance in vitro, even by using the first-line therapy that should be responsible for the least resistance. It includes the MAPK together with PI3K-Akt pathways by overproduction of MAPK or hyperphosphorylation in Akt; NF-κB signaling triggered by over-activated EGFR, associated with cell proliferation and inflammatory response; hypoxia-induced HIF-1α signaling with positive feed-forward loop resulting in higher proliferation and angiogenesis; acquired secondary T790M and C797S that compromise the third generation of EGFR-TKIs from its function. These address the urgency of inhibitor cooperation. Respectfully, a number of inhibitors have been discovered to break the pathways. Dactolisib targets the PI3K but was reported to induce MAPK where normally agonist the PI3K-Akt signaling in NSCLC; RNA interference for NF-κB and HIF-1α inhibitory proteins might fail due to the limitations of the technique itself, such as off-target silencing and inefficient delivery; Bevacizumab, an antibody to re-sensitizing TKIs from secondary mutations off-track from its initiative. Although fourth-generation EGFR-TKI is emerging, the requirement of optimization is granted to be necessary. A new target is aroused by the crosstalking of HIF-1α with other pathways, indicating a mimic or agonist for either hydroxylase or pVHL could be therapeutically promising, which might constantly overcome the drug resistance and benefit the NSCLC patients with EGFR mutations and conducting TKI treatments.

Despite that, considering a founded gap as the target for inhibition, which aims for resistance remission, limited investigation has been done for the agonist and mimicry of hydroxylases and pVHL of NSCLC, and the possibility of

a successful drug candidate and target feasibility remains uncovered. Future studies could focus on searching for drug candidates that are used in other cancerous models. Moreover, validations that follow the drug discovery pipeline require a huge amount of effort and costs, which have not yet been discussed. This includes the optimization of the target, conducting some mutagenesis tests to distinguish the conserved site and flexible regions, and insight into how to make it more soluble, then conducting further in vitro and in vivo trials for efficacy, toxicity, and systemic PK testing. It can be considered after validating the target's efficacy.

References

[1]Fu K., Xie FC., Wang F., et al. Therapeutic strategies for EGFR-mutated non-small cell lung cancer patients with osimertinib resistance. Journal of Hematology & Oncology, 2022, 15(1).

[2]Chmielecki J., Gray JE., Cheng Y., et al. Candidate mechanisms of acquired resistance to first-line osimertinib in EGFR-mutated advanced non-small cell lung cancer. Nature Communications, 2023, 14(1).

[3]Chen CJ., Liu YP. MERTK Inhibition: Potential as a Treatment Strategy in EGFR Tyrosine Kinase Inhibitor-Resistant Non-Small Cell Lung Cancer. Pharmaceuticals, 2021, 14(2).

[4]Eberlein CA., Stetson D., Markovets AA., et al. Acquired Resistance to the Mutant-Selective EGFR Inhibitor AZD9291 Is Associated with Increased Dependence on RAS Signaling in Preclinical Models. Cancer Research, 2015, 75(12): 2489-2500.

[5]Wang LH., Dong XY., Ren Y., et al. Targeting EHMT2 reverses EGFR-TKI resistance in NSCLC by epigenetically regulating the PTEN/AKT signaling pathway. Cell Death & Disease, 2018, 9.

[6]Seguin LJ., Kato S., Franovic A., et al. 3 integrin/KRAS/RalB complex drives tumor stemness and resistance to EGFR inhibition. Cancer Research, 2014, 74(19).

[7]Blakely CM., Pazarentzos E., Olivas,V., et al. NF-B-Activating Complex Engaged in Response to EGFR Oncogene Inhibition Drives Tumor Cell Survival and Residual Disease in Lung Cancer. Cell Reports, 2015, 11(1): 98-110.

[8]Kane LP., Stokoe D., Weiss A. Induction of NF-B by the Akt PKB kinase. Current Biology,1999, 9(11): 601-604.

[9]Rotow J., Bivona TG. Understanding and targeting resistance mechanisms in NSCLC. Nature Reviews Cancer, 2017, 17(11): 637-658.

[10]Meng SY., Wang GR., Lu Y., et al. Functional cooperation between HIF-1 and c-Jun in mediating primary and acquired resistance to gefitinib in NSCLC cells with activating mutation of EGFR. Lung Cancer, 2018, 121: 82-90.

[11]Hu H., Miao XK., Li JY., et al. YC-1 potentiates the antitumor activity of gefitinib by inhibiting HIF-1 and promoting the endocytic trafficking and degradation of EGFR in gefitinib-resistant non-small-cell lung cancer cells. European Journal of Pharmacology, 2020, 874.

[12]Yu HA., Arcila ME., Rekhtman N., et al. Analysis of Tumor Specimens at the Time of Acquired Resistance to EGFR-TKI Therapy in 155 Patients with EGFR-Mutant Lung Cancers. Clinical Cancer Research, 2013, 19(8): 2240-2247.

[13]Nishino M., Suda K., Kobayash Y., et al. Effects of secondary EGFR mutations on resistance against upfront osimertinib in cells with EGFR-activating mutations in vitro. Lung Cancer, 2019, 126: 149-155.

[14]Soria JC., Ohe Y., Vansteenkiste J., Reungwetwattana, T., et al. Osimertinib in Untreated EGFR-Mutated Advanced Non-Small-Cell Lung Cancer. New England Journal of Medicine, 2018, 378(2): 113-125.

[15]Ichihara E., Westover D., Meador CB., et al. SFK/FAK Signaling Attenuates Osimertinib Efficacy in Both Drug-Sensitive and Drug-Resistant Models of EGFR-Mutant Lung Cancer. Cancer Research, 2017, 77(11): 2990-3000.

[16]Zhang X., Maity TK., Ross KE., et al. Alterations in the Global Proteome and Phosphoproteome in Third Generation EGFR TKI Resistance Reveal Drug Targets to Circumvent Resistance. Cancer Research, 2021, 81(11): 3051-3066.

[17]Liu YN., Tsai MF., Wu SG., et al. miR-146b-5p Enhances the Sensitivity of NSCLC to EGFR Tyrosine Kinase Inhibitors by Regulating the IRAK1/NF-B Pathway. Molecular Therapy-Nucleic Acids, 2020, 22: 471-483.

[18]Huang JX., Zhuang C., Chen J., et al. Targeted Drug/Gene/Photodynamic Therapy via a Stimuli-Responsive Dendritic-Polymer-Based Nanococktail for Treatment of EGFR-TKI-Resistant Non-Small-Cell Lung Cancer. Advanced Materials, 2022, 34(27).

[19]Sooy RA., Han JY., Dafni U., et al. A randomised phase II study of osimertinib and bevacizumab versus osimertinib alone as second-line targeted treatment in advanced NSCLC with confirmed EGFR and acquired T790M mutations: the European Thoracic Oncology Platform (ETOP 10-16) BOOSTER trial. Annals of Oncology, 2022, 33(2): 181-192.

[20]Westover D., Zugazagoitia J., Cho BC., et al. Mechanisms of acquired resistance to first- and second-generation EGFR tyrosine kinase inhibitors. Annals of Oncology, 2018, 29: I10-I19.

[21]Wang SH., Song YP., Liu DL. EAI045: The fourth-generation EGFR inhibitor overcoming T790M and C797S resistance. Cancer Letters, 2017, 385: 51-54.

[22]Tang YY., Wang DC., Wang YQ., et al. Emerging role of hypoxia-inducible factor-1 in inflammatory autoimmune diseases: A comprehensive review. Frontiers in Immunology, 2023, 13.

[23]Li PF., Sun Q., Bai SP., et al. Combination of the cuproptosis inducer disulfiram and anti-PD-L1 abolishes NSCLC resistance by ATP7B to regulate the HIF-1 signaling pathway. International Journal of Molecular Medicine, 2024, 53(2).