The Innovation

Review

Acquired resistance to third-generation EGFR-TKIs and emerging next-generation EGFR inhibitors

Xiaojing Du,[1,2,3] Biwei Yang,[1,2] Quanlin An,[1] Yehuda G. Assaraf,[4,*] Xin Cao,[1,*] and Jinglin Xia[1,2,3,5,*]
*Correspondence: assaraf@technion.ac.il (Y.G.A.); caox@fudan.edu.cn (X.C.); xiajinglin@fudan.edu.cn (J.X.)
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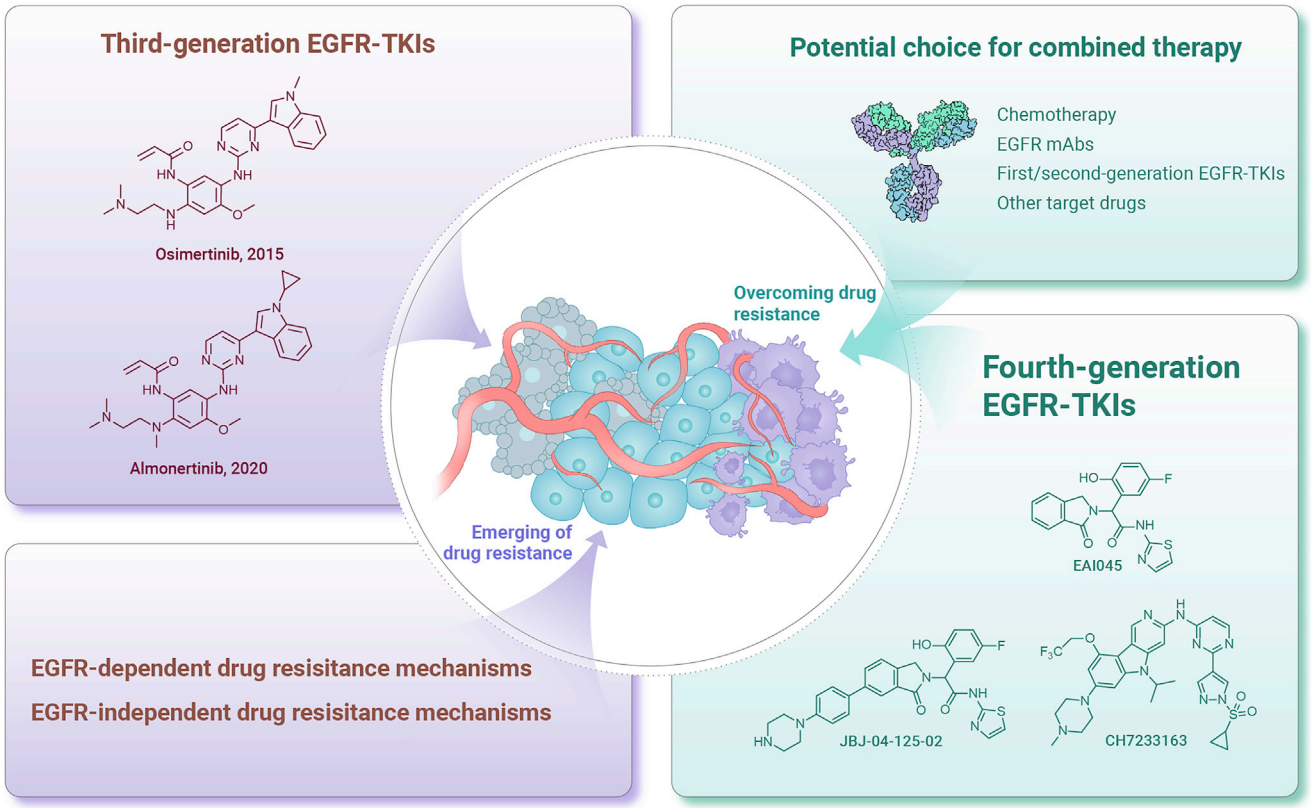
Graphical abstract



Public summary

- *EGFR* gene mutations are detected in about 50% of non-small cell lung cancer (NSCLC) patients worldwide
- The three generations of EGFR tyrosine kinase inhibitors (TKIs) are critical milestones for NSCLC patients
- Like other targeted therapies, new EGFR mutations and coupled drug resistances emerge rapidly after TKI treatment, posing formidable obstacles to cancer management
- The investigational fourth-generation EGFR inhibitors are of great promise, through a number of novel mechanisms, in overcoming these resistances after third-generation TKI treatment, and will bring more benefits to NSCLC patients

Review

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Xiaojing Du,[1,2,3] Biwei Yang,[1,2] Quanlin An,[1] Yehuda G. Assaraf,[4,*] Xin Cao,[1,*] and Jinglin Xia[1,2,3,5,*]

[1]Institute of Clinical Science, Zhongshan Hospital, Fudan University, Shanghai 200032, China

[2]Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai 200032, China

[3]Institute of Fudan-Minhang Academic Health System, Minhang Hospital, Fudan University, Shanghai 201199, China

[4]The Fred Wyszkowski Cancer Research Lab, Department of Biology, Technion-Israel Institute of Technology, Haifa 3200000, Israel

[5]The First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325000, China

*Correspondence: assaraf@technion.ac.il (Y.G.A.); caox@fudan.edu.cn (X.C.); xiajinglin@fudan.edu.cn (J.X.)

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The discovery that mutations in the *EGFR* gene are detected in up to 50% of lung adenocarcinoma patients, along with the development of highly efficacious epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), has revolutionized the treatment of this frequently occurring lung malignancy. Indeed, the clinical success of these TKIs constitutes a critical milestone in targeted cancer therapy. Three generations of EGFR-TKIs are currently approved for the treatment of EGFR mutation-positive non-small cell lung cancer (NSCLC). The first-generation TKIs include erlotinib, gefitinib, lapatinib, and icotinib; the second-generation ErbB family blockers include afatinib, neratinib, and dacomitinib; whereas osimertinib, approved by the FDA on 2015, is a third-generation TKI targeting EGFR harboring specific mutations. Compared with the first- and second-generation TKIs, third-generation EGFR inhibitors display a significant advantage in terms of patient survival. For example, the median overall survival in NSCLC patients receiving osimertinib reached 38.6 months. Unfortunately, however, like other targeted therapies, new EGFR mutations, as well as additional drug-resistance mechanisms emerge rapidly after treatment, posing formidable obstacles to cancer therapeutics aimed at surmounting this chemoresistance. In this review, we summarize the molecular mechanisms underlying resistance to third-generation EGFR inhibitors and the ongoing efforts to address and overcome this chemoresistance. We also discuss the current status of fourth-generation EGFR inhibitors, which are of great value in overcoming resistance to EGFR inhibitors that appear to have greater therapeutic benefits in the clinic.

Keywords: Cancer; targeted therapy; EGFR inhibitors; drug resistance; mutations; chemoresistance mechanisms; surmounting chemoresistance

INTRODUCTION

Mutated epidermal growth factor receptor (EGFR) is a very common driver oncogene and hence constitutes a central druggable target in cancer treatment.[1] The first EGFR inhibitor, gefitinib, launched in 2003, marked the beginning of a new era in cancer treatment, i.e., targeted therapy.[2] Great progress has been made in the treatment of non-small cell lung cancer (NSCLC), which includes EGFR-targeted tyrosine kinase inhibitors (TKIs).[2] Unfortunately, however, NSCLC patients treated with EGFR inhibitors frequently develop anticancer drug resistance after months of treatment, frequently via EGFR mutations and other molecular mechanisms of chemoresistance.[3] It should be emphasized that both intrinsic (primary) and acquired (secondary) anticancer drug resistance frequently emerges in various cancers and constitute a central impediment to curative cancer therapy.[4–11] To address and overcome this major impediment, new EGFR inhibitors are being developed that target some of these resistance-associated EGFR mutations. Accompanied by the first-line therapy of the third-generation EGFR tyrosine kinase inhibitors (EGFR-TKIs), especially the great progress of osimertinib in treating NSCLC patients, new EGFR mutations as well as drug-resistance mechanisms have emerged.[12,13] In this review, we summarize the highly heterogeneous molecular mechanisms that mediate resistance of cancers harboring mutant EGFR to third-generation EGFR-TKIs. We also discuss recent progress in the development of the fourth-generation EGFR inhibitors, which are of great value in overcoming the emerging EGFR inhibitor resistance and which appear to achieve greater therapeutic benefits in cancer patients.

EGFR PATHWAY IN CANCER AND LAUNCHED EGFR INHIBITORS

The EGFR family harbors an extracellular domain, a transmembrane region, and an intracellular domain.[1] The intracellular kinase domain contains the N-lobe, which is subdivided into nucleotide-binding site, P-loop, C-helix, and C-lobe, including the DFG motif, catalytic residue Asp813, catalytic loop, and A-loop, with the ATP-binding pocket located in the catalytic cleft between the lobes.[1] The A-loop folds into a helix under the inactive state and restrains C-helix rotation toward the catalytic cleft (C-helix-out; Figure 1A).[1,14] When triggered by ligands, EGFR signal acts an asymmetric dimer, following which the kinase domain turns into a tail-to-head interaction, which shifts the equilibrium to an active state by pushing the C-helix into an active position (C-helix-in; Figure 1B),[15] thereby resulting in the subsequent activation of PI3K/AKT, RAS/RAF/MEK/ERK, and STAT3 signaling pathways.[1] These pathways regulate pivotal cellular processes, including proliferation, differentiation, survival, and migration, making EGFR signaling susceptible to being hijacked by cancer cells.[1,2] Various pre-clinical and clinical studies to date have revealed the driver function of EGFR in various cancer types.[1,2]

The first-generation EGFR-TKIs (Figure 2), including gefitinib, erlotinib, lapatinib, and icotinib, have been widely used to block EGFR activity in an ATP-competitive and -reversible manner.[2] Their cytotoxic effect has been stronger when targeting mutated EGFR than wild-type EGFR ($EGFR^{WT}$), especially EGFR harboring an L858R mutation as well as EGFR displaying exon19 deletion (Ex19del).[2] However, almost all cancer patients developed resistance to the first-generation EGFR-TKIs after 10–14 months of treatment; this TKI resistance has been associated with the EGFR T790M mutation in 50%–60% of the cases.[16]

Over the years, second-generation EGFR-TKIs with more potent inhibitory activity against EGFR have been developed and applied.[2] Covalent binding of these TKIs to EGFR at Cys797 residue may lead to irreversible inhibition of the EGFR kinase.[2] The second-generation EGFR-TKIs afatinib, neratinib, and dacomitinib have shown a higher antitumor activity compared with the first-generation EGFR-TKIs.[2] Unfortunately, these EGFR-TKIs continue to be limited in their activity due to the frequent emergence of the drug-resistance

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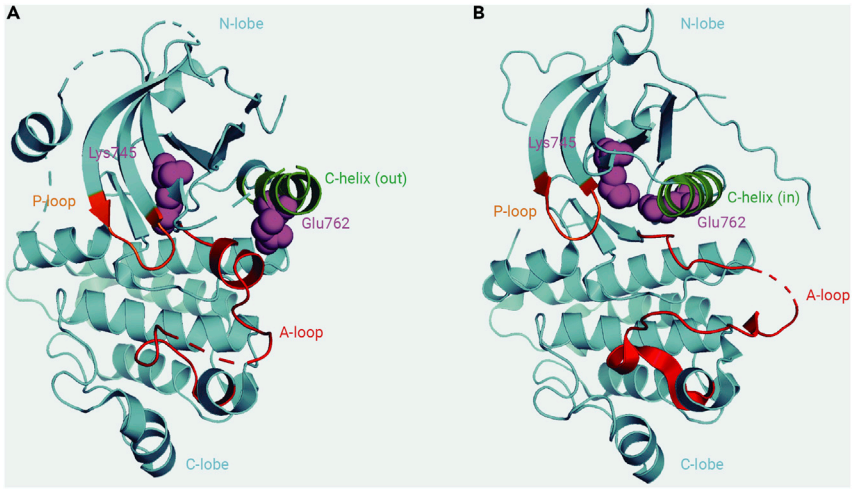

Figure 1. Conformational movement occurs in human EGFR tyrosine kinase domains between inactive and active states (A and B) Crystal structure of the inactive state (PDB: 1XKK) and active state of the EGFR tyrosine kinase domain (PDB: 2ITX) were visualized by using PyMol 2.4.1. When in the inactive conformation, residues Lys745 and Glu762 are separated, the C-helix is in the out conformation, and the A-loop is in its closed conformation. When active, residues Lys745 and Glu762 are adjacent, the C-helix is in the in conformation, and the A-loop is in its open conformation.

phenomenon (especially when targeting the T790M EGFR mutation); moreover, an irreversible mode of EGFR inhibition may lead to increased side effects.[2]

Osimertinib, which is the most successful third-generation EGFR-TKI, was approved by the US Food and Drug Administration (FDA) in 2015 for the treatment of locally advanced or metastatic NSCLC bearing the T790M EGFR mutation or other activating mutations.[12,17–22] Osimertinib irreversibly binds to Cys797 in the EGFR kinase domain and has a stronger antitumor activity compared with other EGFR-TKIs.[12,17,18,23] Moreover, osimertinib can overcome T790M mutation-induced resistance.[12,23] Other promising third-generation EGFR-TKIs include almonertinib, maverlertinib, nazartinib, avitinib, ASK120067, rociletinib, olmutinib, naquotinib, and lazertinib, most of which have been tested in pre-clinical and clinic trials (Table 1).[2,24] In addition, multiple-targeted TKIs, such as vandetanib, brigatinib, and pyrotinib, also suppress EGFR activity and have been applied in the clinic.[2,28]

EGFR exon 20 insertion (Ex20ins) mutations account for 4%–10% of EGFR mutations.[29] Apart from the insertion mutation 763_764insFQEA, most exon 20 insertion mutations are resistant to first- and second-generation EGFR-TKIs.[29,30] The efficacy of the third-generation EGFR-TKIs on Ex20ins mutations is still controversial.[31] Currently, chemotherapy is preferred for these mutations in the clinic.[32,33] To overcome these EGFR mutations, several TKIs targeting these mutations have been trialed. TAK-788 (AP32788) targets 14 distinct Ex20ins mutations in EGFR and six types of Ex20ins mutations in human epidermal growth factor receptor2 (HER2).[34] Data from a phase I/II study affirmed the effectiveness of TAK-788 in NSCLC patients harboring Ex20ins mutations.[35] Amivantamab (JNJ-61186372) is an EGFR/cMET targeted bispecific antibody.[36] A phase I study of 20 NSCLC patients with Ex20ins mutations demonstrated that the disease control rate of amivantamab is 100%, and the objective response rate (ORR) was 30%.[37,38] Other agents targeting Ex20ins mutations, including DZD9008 and CLN-081, a pan-mutation-selective EGFR-TKI, also achieved a promising effect in pre-clinical research and merit further research.[39–41]

Four EGFR-targeted monoclonal antibodies (mAbs), cetuximab, necitumumab, panitumumab, and nimotuzumab, have been approved by the FDA.[2] Since the mAbs could suppress the ligand-dependent dimerization of $EGFR^{WT}$ and some mutant EGFR via binding to the extracellular domain of EGFR that competes for endogenous ligand binding, they are sometimes also classified as EGFR inhibitors. Combined administration with chemotherapeutic agents is frequently required for EGFR mAbs in the clinic.[2] It is noteworthy that only a fraction of the patients is sensitive to these mAbs, and $EGFR^{WT}$-targeted toxicities may restrict their clinical application.[2]

EGFR-TARGETED THERAPY IN THE CLINIC

The cellular proliferation features of EGFR render it heavily implicated in malignant tumors; this has been confirmed by a large amount of pre-clinical and clinical evidence.[2,7,42–44] The majority of aberrant forms of EGFR in malignant tumors emerge as mutations and/or gene amplification, which has been reported to occur in lung cancer,[45–47] colorectal cancer,[48,49] squamous cell carcinoma of the head and neck (SCCHN),[50,51] pancreatic cancer,[52–54] renal cell cancer,[55] hepatocellular carcinoma,[56] breast cancer,[57–59] prostate cancer,[60–62] gastric cancer,[63,64] glioma,[65–67] and ovarian cancer,[68,69] and others (Table 2). Among these malignancies, the involvement of EGFR in lung cancer, especially NSCLC, has received the most research attention.

Lung cancer

EGFR is a major driver gene in NSCLC, the most common lung cancer.[7] Precise molecular typing is highly recommended before administration of EGFR-targeted therapy according to the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology.[33] For patients with the common sensitive EGFR mutations (Ex19del and L858R), osimertinib is recommended.[33] The FLAURA study on advanced NSCLC patients with the common sensitive EGFR mutations showed that the median progression-free survival (mPFS) was 10.2 months for the first-generation EGFR-TKIs (gefitinib and erlotinib) and 18.9 months for osimertinib (hazard ratio [HR] = 0.46, $p < 0.001$).[17,18] Moreover, the median duration of response (mDoR) was 8.5 months and 17.2 months, and the median overall survival (mOS) was 31.8 months and 38.6 months (HR = 0.8, p = 0.046), respectively.[17,18] At 3 years, 28% of patients in the osimertinib group and 9% in the gefitinib and erlotinib group continued with a clinical trial.[18] Osimertinib also has a similar safety profile and lower rates of severe adverse events than the standard EGFR-TKIs.[17] Combined post hoc analysis of LUX-Lung 2, LUX-Lung 3, and LUX-Lung 6 clinical trials revealed that afatinib was active in patients with uncommon EGFR mutations, especially G719X, L861G, and S768I.[70] The mPFS and mOS values for afatinib in the three groups of EGFR mutations were 10.7, 2.9, and 2.7 months and 19.4, 14.9, and 9.2 months, respectively.[70] Another phase II study (KCSG-LU15-09) showed that osimertinib demonstrated favorable activity with manageable toxicity in patients with NSCLC harboring uncommon EGFR mutations; the ORR, mPFS, and mDoR for osimertinib were 50%, 8.9 months, and 11.2 months, respectively.[19] However, so far no high-quality randomized controlled trial (RCT) has directly compared the efficacy of osimertinib and the second-generation EGFR-TKIs in NSCLC patients harboring uncommon EGFR mutations.

For patients with advanced NSCLC with central nervous system (CNS) metastases, the first-/second-generation EGFR-TKIs were ineffective, since the penetration of these drugs into the brain is relatively low.[20] In

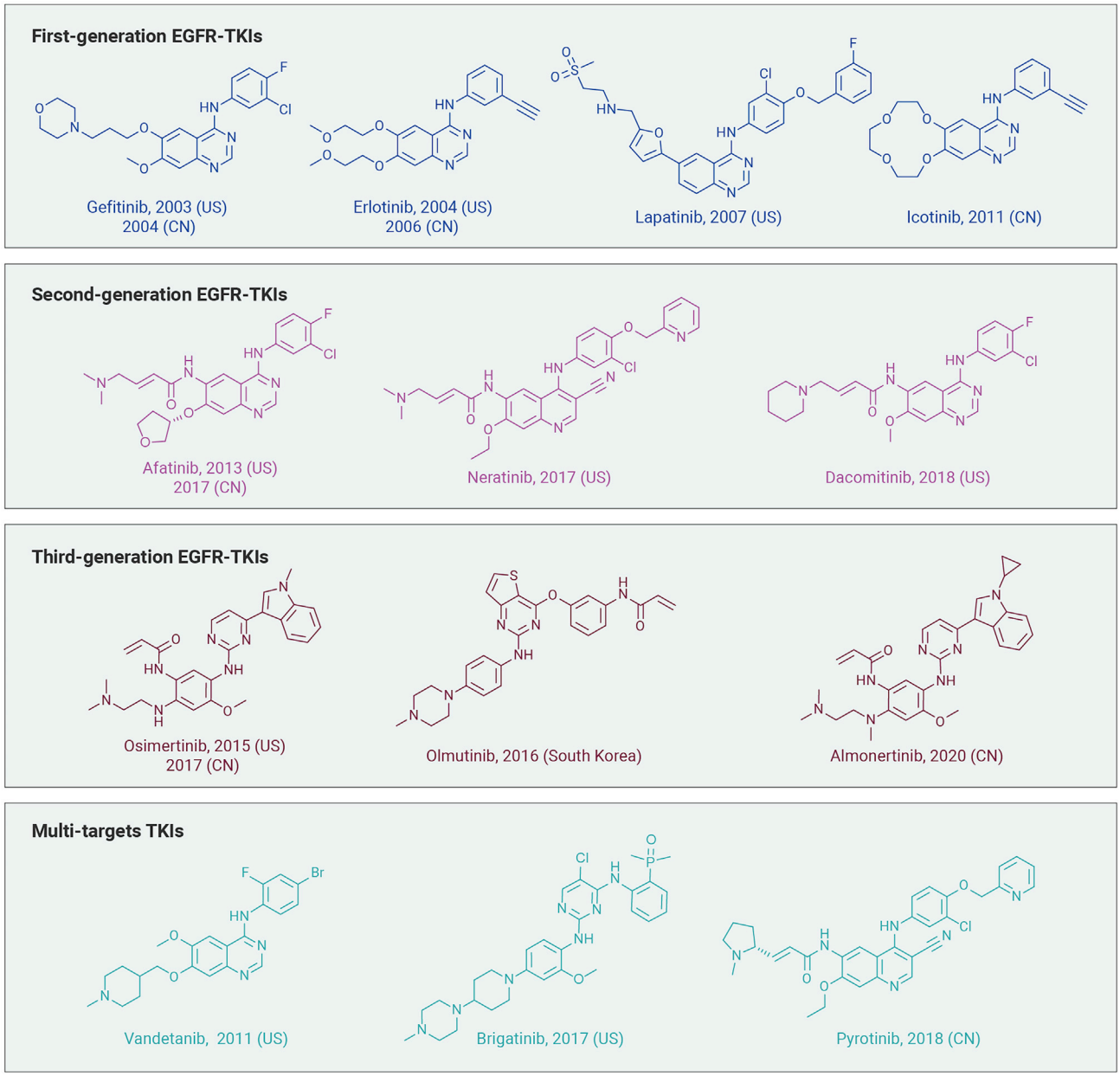

Figure 2. Global launched EGFR inhibitors The launching time and chemical structures of three generations of EGFR-TKIs are displayed. Moreover, the related multi-targeted TKIs are also listed for reference. CN, China; US, United States.

contrast, osimertinib effectively crossed the blood-brain barrier (BBB) due to its lower efflux by human BBB multi-drug efflux pumps than other EGFR-TKIs, which implies the attainment of a higher concentration of osimertinib in the brain.[71] The AURA3 study on CNS metastasis in NSCLC patients with the T790M EGFR mutation demonstrated that CNS ORR was 70% with osimertinib and only 31% with the platinum-pemetrexed combination (odds ratio [OR] = 5.13, p = 0.015); the ORR was 40% and 17% (OR = 3.24, p = 0.014), respectively.[20] The mDoR of CNS was 8.9 months for osimertinib and 5.7 months for platinum-pemetrexed, whereas the mPFS of CNS was 11.7 and 5.6 months (HR = 0.32, p = 0.004), respectively.[20] Moreover, the BLOOM study on EGFR-mutated NSCLC patients with leptomeningeal metastases showed that mPFS and mOS of osimertinib were 8.6 months and 11.0 months, respectively.[21] Among these patients, the neurologic function was ameliorated in 57% (12/21) of patients with an abnormal assessment at baseline, which improved the quality of life and prognosis.[21]

For patients with advanced NSCLC whose sensitive EGFR mutations were found during chemotherapy, completion or interruption of the planned chemotherapeutic regimen was recommended, followed by an administration of osimertinib or the first-/second-generation EGFR-TKIs.[33] When the disease progresses after osimertinib treatment, definitive local therapy combined with continued treatment of osimertinib is recommended for asymptomatic patients and patients with brain metastases or isolated lesions.[33] When the disease progresses after other EGFR-TKI treatment, the detection of the T790M mutation is recommended, and osimertinib is the preferred treatment for patients with this frequent T790M EGFR mutation.[33] As postoperative adjuvant therapy, osimertinib significantly reduces the recurrence rate after the operation by 79%.[22]

Colorectal cancer

EGFR is a crucial target for colorectal cancer. EGFR mAbs are the first group of targeted drugs developed for the treatment of metastatic colorectal

Table 1. Clinical progress of the third-generation EGFR-TKIs

Drugs	Stage	Company	Indications	Clinical trials	Refs.
Osimertinib	2015, approved (US); 2017, approved (CN)	AstraZeneca	NSCLC, other solid cancers	NCT02296125[a], NCT02151981[a], NCT02511106[a], NCT03521154[a], NCT04413201[a]	Soria et al.[17], Ramalingam et al.[18], Cho et al.[19], Wu et al.[20], Yang et al.[21], Wu et al.[22]
Olmutinib	2016, approved (South Korea)	Hanmi Pharma	NSCLC	NCT01588145[a], NCT02485652[a], NCT03228277[a]	Guardiola et al.[2], Wang and Adjei[24], Tan et al.[25]
Almonertinib	2020, approved (CN)	Hansoh Pharma	NSCLC	NCT04500717[a], NCT04500704[a]	Wang and Adjei[24]
Maverlertinib	phase II	Pfizer	NSCLC	NCT02349633[a],	Wang and Adjei[24], Tan et al.[25]
Nazartinib	phase II	Novartis	NSCLC, other solid cancers	NCT02335944[a], NCT03040973[a], NCT02323126[a]	Wang and Adjei[24], Tan et al.[25]
Avitinib	phase II	ACEA Pharma	NSCLC, B cell lymphoma	NCT03300115[a], NCT03060850[a]	Wang and Adjei[24], Tan et al.[25]
CK-101	phase II	CKPT	NSCLC, other solid cancers	NCT02926768[a]	Wang and Adjei[24]
D-0316	phase II	InventisBio	NSCLC, other solid cancers	NCT03861156[a], NCT04206072[a]	Wang and Adjei[24]
BPI-7711	phase III	Beta Pharma	NSCLC	NCT03866499[a]	Wang and Adjei[24]
SH-1028	phase III	Sanhome Pharma	NSCLC	NCT04239833[a]	Wang and Adjei[24]
ASK120067	phase III	Aosaikang Pharma	NSCLC	NCT04143607[a]	Wang and Adjei[24], Zhang et al.[26]
ZN-e4	phase II	Zeno Pharma	NSCLC	NCT03446417[a]	Wang and Adjei[24]
Rociletinib	phase III	Clovis Oncol	NSCLC	NCT02147990[a]	Wang and Adjei[24], Tan et al.[25]
Naquotinib	phase I	Astellas Pharma	NSCLC	NCT02113813[a]	Wang and Adjei[24], Tan et al.[25]
Lazertinib	phase III	Yuhan/Genosco/ Janssen Pharma	NSCLC	NCT04248829[a]	Wang and Adjei[24]
				NCT04487080[a]	
Alflutinib	phase III	Allist Pharma	NSCLC	NCT03787992[a]	Wang and Adjei[24]
ES-072	phase I	Bossan	NSCLC	CTR20180074[b]	
TY-9591	phase I	TYK Med	NSCLC	NCT04204473[a]	
TAS-121	phase I	Taiho Pharma	NSCLC	JapicCTI142651[c]	
BPI15086	phase I	Beta Pharma	NSCLC	NCT02914990[a]	
FHND9041	phase II	Chuangte Pharma	NSCLC	CTR20191359[b]	
C-005	phase I	Shuangliang Bio	NSCLC	CTR20191910[b]	
BEBT-109	phase I	Bebetter Med	NSCLC	CTR20192575[b]	
YZJ-0318	phase I	Haiyan Pharma	NSCLC	CTR20171646[b]	
TQB3456	phase I	ChiaTai TianQing	NSCLC	NCT03754244[a]	Wang and Adjei[24]
WZ4002	pre-clinical				Ricordel et al.[27]
CNX-2006	pre-clinical				Ricordel et al.[27]

CN, China; US, United States; CKPT, Checkpoint Therapeutics; NSCLC, non-small cell lung cancer.
[a]https://www.clinicaltrials.gov
[b]http://www.chinadrugtrials.org.cn
[c]https://www.clinicaltrials.jp

cancer (mCRC). In patients with wild-type KRAS, Cetuximab combined with FOLFIRI significantly improved mOS (23.5 versus 20.0 months; HR = 0.796, p = 0.0093), mPFS (9.9 versus 8.4 months; HR = 0.696, p = 0.0012), and response rate (RR) (57.3% versus 39.7%; OR = 2.069, p = 0.001) when compared with FOLFIRI alone.[72] Addition of cetuximab to FOLFOX4 remarkably prolonged mPFS (8.3 versus 7.2 months, HR = 0.576, p = 0.0064) than FOLFOX4 alone, resulting in a trend of OS benefit (22.8 versus 18.5 months, HR = 0.855, p = 0.39).[73] Cetuximab plus panitumumab also improved the mPFS of FOLFOX4 (10.0 versus 8.6 months, HR = 0.80, p = 0.01).[74] Interestingly, mCRC originating from the left colon was found to be more sensitive to

Table 2. Aberrant EGFR in different cancers

Cancer type	EGFR aberration	EGFR inhibitors approved by FDA in clinic
NSCLC	15%–50% mutation (Ex19del, L858R, Ex20ins, G719X, L861G, S768I, E709X, etc.),[46,47] 32% gene amplification[45]	erlotinib, gefitinib, afatinib, dacomitinib, osimertinib, brigatinib[a]
Colorectal cancer	7%–89% increased gene copy number,[49] <1% mutation[48]	cetuximab, panitumumab
SCCHN	10%–17% gene amplification,[50] 42% EGFRvIII mutation[51]	cetuximab, nimotuzumab
Pancreatic cancer	55%–69% protein overexpression,[52,53] 1.5% mutation[54]	erlotinib
Renal cell cancer	38% gene amplification[55]	NA
Hepato carcinoma	68% protein overexpression[56]	NA
Breast cancer	14%–64% gene amplification,[57,58] 3%–11% mutation[58,59] (Ex19del, T847I, L858R, G719A, V786M), 13% SNP (T725T, Q787Q)[59]	lapatinib, neratinib, pyrotinib, afatinib
Prostate cancer	14%–30% protein overexpression,[60,61] 15% mutation[62] (Ex19del, V738G, D761G, E709K)	NA
Gastric cancer	27% protein overexpression,[63] 5.2% mutation[64] (Y801C, L858R, G863D); 37.9% SNP[64] (Q787Q)	NA
Glioma	40%–45% gene amplification,[65] 14% mutation[66,67] (D46N, G63R, R108K, T263P, A289X, R324L, E330K, P596L, G598V, L861Q); 30% EGFRvIII mutation[52]	nimotuzumab
Ovarian cancer	4%–22% gene amplification,[68] 23.5% mutation[69] (Ex19del, T725X, L858R, R832C, E868D, T852M, L703P, S720F, N700S, etc.)	NA

[a]The approval of brigatinib for NSCLC is mainly due to its ALK inhibition.

EGFR-targeted mAbs.[75] Hence, the first-line treatment of combined EGFR mAbs with chemotherapy is recommended only for metastatic colon cancer with wild-type KRAS, NRAS, or BRAF in the clinic.[76] Another phase II study revealed that cetuximab plus erlotinib was also active in mCRC with wild-type KRAS (mPFS = 5.6 months), hence deserving further clinical assessment.[77]

Squamous cell carcinoma of the head and neck

Cetuximab was the first mAb that showed clinical activity for SCCHN. Cetuximab combined with radiotherapy notably ameliorated the prognosis of SCCHN.[78] The mOS times in the group of this cetuximab and radiotherapy group and the group undergoing radiotherapy alone were 49.0 and 29.3 months (HR = 0.74, p = 0.03), mPFS was 17.1 and 12.4 months (HR = 0.70, p = 0.006), and the 5-year survival rate was 45.6% and 36.4%, respectively.[78] Erlotinib, combined with cisplatin and radiotherapy in patients with locally advanced SCCHN, failed to improve PFS.[79] Multi-targeted EGFR-TKIs such as afatinib, dacomitinib, and lapatinib may be effective in SCCHN, although they should also be further assessed for their potential side effects.[2] In recurrent metastatic nasopharyngeal carcinoma after radical radiotherapy, addition of nimotuzumab to chemotherapy resulted in a superior ORR, PFS, and OS than those who did not receive this nimotuzumab addition (88.9% versus 12.5%, p < 0.001; 7.4 versus 2.7 months, p = 0.081; 17.0 versus 8.0 months, p = 0.202).[80] This combination was well tolerated and displayed a potential clinical application prospect in nasopharyngeal carcinoma.[80]

Pancreatic cancer

Combining erlotinib with gemcitabine can improve the prognosis of pancreatic cancer patients, and this combination has been clinically approved.[81] A phase II study of advanced pancreatic cancer with wild-type KRAS showed that nimotuzumab plus gemcitabine could significantly ameliorate patient prognosis.[82] The mOS for combined therapy and gemcitabine alone was 8.6 and 6.0 months (HR = 0.69, p = 0.0341), mPFS was 5.1 and 3.4 months (HR = 0.68, p = 0.0163), and the 1-year OS/PFS rate was 34%–22% and 19%–10% (HR = 0.69, p = 0.03; HR = 0.68, p = 0.02), respectively.[82] Neoadjuvant therapy with intensity-modulated radiotherapy, cetuximab, and gemcitabine was tolerable and increased margin negative resection rates.[83] Partial locally advanced tumors may be downstaged by applying this combination to allow for complete resection.[83] However, the addition of cetuximab to gemcitabine could not improve prognosis compared with gemcitabine alone, and EGFR inhibitors plus MEK inhibitors were also of finite benefit to advanced pancreatic cancer.[84,85] Overall, although EGFR-targeted therapy can improve pancreatic cancer prognosis, its clinical benefit is still limited.

Renal cell cancer

A phase II study showed that erlotinib improved disease control and survival outcomes under acceptable toxicity in renal cell cancer, with mOS of 27 months.[86] Unfortunately, gene detection was absent before treatment.[86] Detailed molecular typing may enhance the efficacy of EGFR inhibitors in renal cell cancer. Other clinical trials suggested that erlotinib plus bevacizumab achieved mPFS of 11 months in metastatic clear renal cell cancer.[87] Lapatinib resulted in longer OS than hormone in EGFR-positive advanced renal cell cancer (46.0 versus 37.9 weeks, HR = 0.69, p = 0.02).[88] These clinical trials highlight the therapeutic potential of EGFR inhibitors in renal cell cancer; however, additional investigations are warranted.

Hepatocellular carcinoma

A phase II study of patients with advanced hepatocellular carcinoma suggested that the mOS of erlotinib was 13 months.[89] Combined erlotinib with bevacizumab achieved more potent activity, prolonging mPFS and mOS to 9 and 15.7 months, respectively.[90] Another phase II study found that lapatinib was well tolerated, while it only had a therapeutic effect on a certain subgroup of patients with advanced hepatocellular carcinoma (mPFS = 1.9 months; mOS = 12.6 months), for whom predictive molecular or clinical characteristics have not been fully clarified.[91]

Breast cancer

HER2 is the main driver gene in breast cancer. EGFR/HER2 target EGFR-TKIs, such as pyrotinib, neratinib, afatinib, and lapatinib, display an efficacy in breast cancer.[2] For selective EGFR-TKIs, data from a phase II study uncovered a finite efficacy of erlotinib plus bevacizumab in breast cancer.[92] Moreover, another study showed that a combination of gefitinib and anastrozole or trastuzumab could not improve the treatment efficacy in breast cancer.[93,94] A phase II study of triple-negative breast cancer showed that addition of cetuximab to cisplatin achieved clinical benefit compared with cisplatin alone; mPFS for cetuximab plus cisplatin was 3.7 months and for cisplatin it was 1.5 months (HR = 0.67, p = 0.032), whereas mOS was 12.9 and 9.4 months (HR = 0.82, p = 0.31), respectively.[95] However, these researches also suggested that the application of selective EGFR inhibitors for breast cancer should be further assessed.

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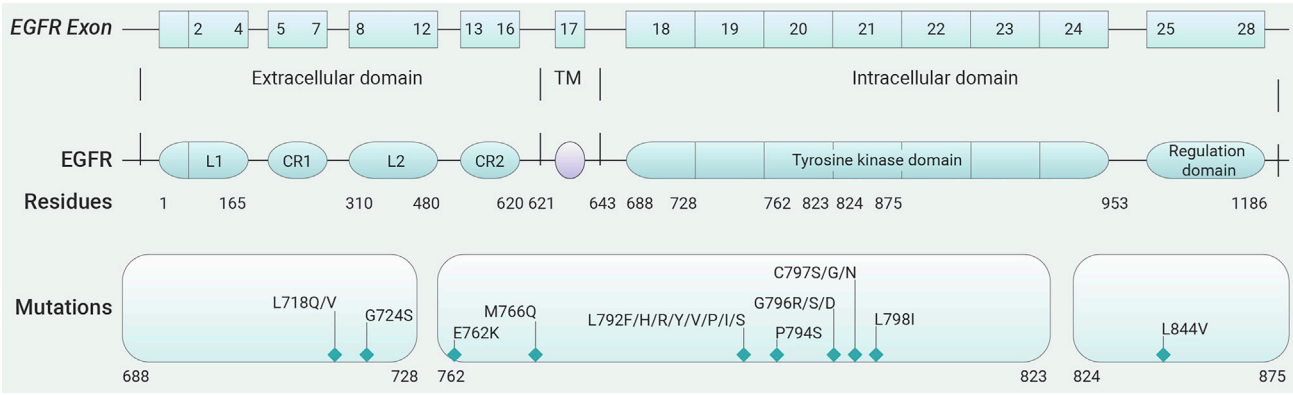

Figure 3. Summary of EGFR mutations associated with resistance to third-generation EGFR-TKIs Shown are the various EGFR mutations that result in resistance to TKIs. The localization of these mutations on the various domains of the EGFR protein and exons are also depicted. L1, L2, ligand-binding domains; CR1, CR2, cysteine-rich domains; TM, transmembrane region.

Prostate cancer

A phase II study of metastatic prostate cancer showed that erlotinib achieved clinical benefit (decrease or stabilization of prostate-specific antigen [PSA] levels without clinical progression) in 40% of the patients.[96] For androgen-resistant metastatic prostate cancer, a phase II study indicated that cetuximab decreased PSA in some patients and prolonged survival time (mOS = 13.3 months).[97]

Other indications

Multi-target EGFR inhibitors have been applied in other cancer types such as gastric cancer, thyroid cancer, leukemia, and ovarian cancer.[2] These EGFR inhibitors may achieve activity by suppressing kinase targets other than EGFR. Nevertheless, selective EGFR inhibitors need further investigation in these cancer types.

MECHANISMS OF ACQUIRED RESISTANCE TO THIRD-GENERATION EGFR-TKIs

Considering the capacity to overcome resistance induced by the T790M EGFR mutation, the third-generation EGFR-TKIs, especially osimertinib, have received a great deal of attention. After approximately 1–2 years of treatment, disease progression occurred in most patients, implying the emergence of a drug-resistant phenotype.[98] Mechanisms of the acquired resistance to the third-generation EGFR-TKIs possess significant tumor heterogeneity and can be classified as EGFR-dependent and EGFR-independent in general (Figures 3, 4, and 5; Table 3).

EGFR-dependent mechanisms

EGFR mutations. EGFR mutations, especially in exons 18, 20, and 21, account for about one-third of the mechanisms of resistance to the third-generation EGFR-TKIs (Figure 4).[25] Cys797 is the most frequent mutation hotspot. Non-Cys797 EGFR mutations often coexist with other EGFR mutations.[13,25] These mutations reduce the binding of the third-generation EGFR-TKIs to EGFR via different mechanisms.

Mutations in EGFR exon 20. C797S (22%–40% of the mutations) is the most frequent mutation that mediates resistance to the third-generation EGFR-TKIs.[25] Cys797 is located at the edge of the ATP-binding pocket in the EGFR kinase domain. Third-generation EGFR-TKIs such as osimertinib and rociletinib are based on a pyrimidine scaffold and covalently bind to Cys797 via a Michael acceptor group.[23,102,131] The C797S mutation prevents the formation of the covalent bond and results in drug resistance.[23,102,131] Clinical data showed that the C797S mutation is more frequent in the Ex19del mutation.[132] Intriguingly, activation of downstream signaling pathways induced by L858R/T790M/C797S mutant EGFR partially requires EGFR dimerization, promoting its sensitivity to cetuximab to a certain extent.[13] For patients without the T790M mutation, C797S mutated EGFR moderately responds to the first-generation EGFR-TKIs.[99] In patients with the T790M mutation, the colocalization of the T790M and C797S on the allele determines the option of a treatment strategy for overcoming C797S-mediated resistance. If C797S and T790M reside on different alleles (T790M/*trans*-C797S mutation), a combination of the first- and third-generation EGFR-TKIs may be active.[100,101] If residing on the same allele (T790M/*cis*-C797S mutation), all clinically approved EGFR inhibitors will fail to surmount drug resistance.[133] To overcome the T790M/C797S mutation, the combination therapy of brigatinib with EGFR mAbs or the mutant-selective allosteric inhibitors may be an effective strategy.[103,104] Some studies pointed out that the C797G or C797N EGFR mutations also mediated resistance to third-generation EGFR-TKIs.[25,134]

Next-generation sequencing (NGS) on cell-free DNA (cfDNA) of 93 osimertinib-resistant NSCLC patients revealed Gly796/Cys797 as well as Leu792 EGFR mutations in 24.7% and 10.8% of the cases, respectively.[135] Among the L792X mutations including L792F, L792H, L792R, L792Y, L792V, and L792P, the L792H EGFR mutation results in the most potent resistance to osimertinib.[135] L792X mutations in the absence of T790M also lead to osimertinib resistance.[135] G796X mutations include G796R and G796S.[135] Structural analyses uncovered that the L792H and G796R mutations sterically and energetically hinder the binding of osimertinib to EGFR via a similar mechanism.[105] The L792H mutation is sensitive to the combination therapy of cetuximab + osimertinib.[105] Moreover, docetaxel, a potent antimitotic cytotoxic agent, effectively decreased the proliferation of tumor cells with L792H or G796S mutation.[105]

cfDNA detection in an L858R/T790M-mutated NSCLC patient who received a third-line treatment of osimertinib revealed that the M766Q mutation apparently induced disease progression.[106] A further study indicated that this M766Q mutation reduced the binding of osimertinib to EGFR, resulting in osimertinib resistance.[106] Cells with this mutation were also resistant to the most first-/second-generation EGFR-TKIs but retained sensitivity to neratinib and poziotinib.[106]

The C797S mutation occurs in <3% of NSCLC patients after rociletinib treatment.[136] A new L798I mutation was found in *cis* with T790M by circulating tumor DNA (ctDNA) analysis.[136] Molecular dynamics simulations revealed that the Asp800 side chain forms a hydrogen bond with the quaternary piperazine NH^+ of rociletinib in the T790M mutation.[136] However, the double T790M/L798I mutation triggered the rotation of rociletinib orientation in EGFR, which hindered the formation of this hydrogen bond, and altered the angle of the Cys797 side chain, which diminished its nucleophilicity, leading to reduction in the binding affinity of rociletinib to EGFR.[136] Importantly, MET gene amplification was found to be the most frequent mechanism of rociletinib resistance, and patients with multiple pre-existing resistance mechanisms (T790M and MET gene amplification) experience inferior responses.[136] Similarly, rociletinib-resistant xenografts develop MET gene amplification that can be overcome with the MET inhibitor crizotinib.[136]

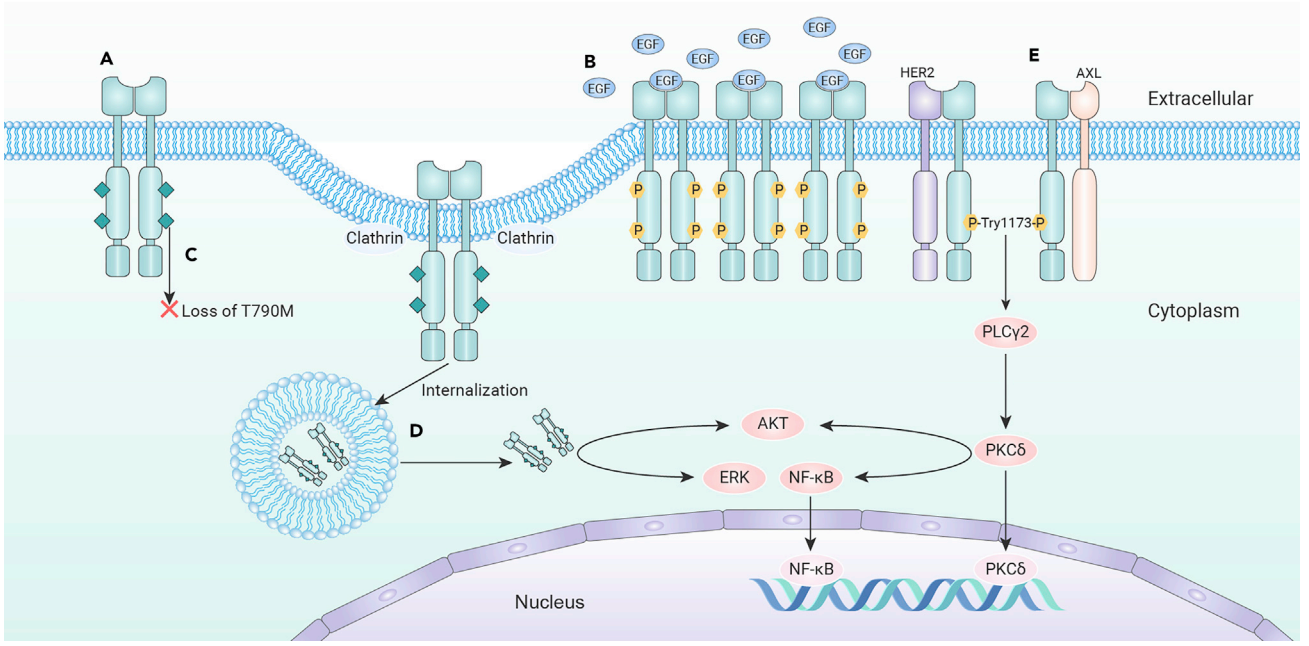

Figure 4. EGFR-dependent mechanisms of resistance to third-generation EGFR-TKIs (A) Mutations located in the EGFR kinase domain induce resistance to third-generation EGFR inhibitors. (B) Amplification of the $EGFR^{WT}$ and EGF. (C) Deletion of the T790M mutation. (D) Clathrin-mediated internalization could not induce the degradation of mutant EGFR. Internalized mutant EGFR still harbors the capacity to activate downstream signaling pathways. (E) Heterodimerization of EGFR and HER2 or AXL activates PKCδ via phosphorylation of Tyr1173. Activated PKCδ triggers nuclear translocation of PKCδ and activates AKT and NF-κB signaling.

Cells with EGFR D770insSVD, H773insH, or H773insNPH mutations retain sensitivity to osimertinib. When these EGFR insertion mutations are accompanied by E762K, L792I/S, P794S, or G796D mutation, tumor cells acquire resistance to osimertinib.[137]

Mutations in EGFR exon 18. The aforementioned analysis of 93 osimertinib-resistant NSCLC patients also found EGFR Leu718/Gly719 mutations in 9.7% of the patients.[135] Leu718 is located in the ATP-binding pocket of the EGFR kinase domain. L718Q results in more robust resistance to osimertinib than the L792X mutation.[135] The L858R/T790M/*cis*-L718Q mutation shows similar resistance to osimertinib compared with the L858R/T790M/*cis*-C797S mutation, but slightly less resistance than the Ex19del/T790M/*cis*-C797S mutation.[135] The L718Q mutation also induces resistance to osimertinib in the absence of the T790M mutation.[135] Free-energy calculations uncovered that the L718Q mutation does not decrease the affinity of osimertinib to EGFR, whereas Gln718 forms a hydrogen bond with an acrylamide warhead of osimertinib and maintains the latter in a specific conformation, which hampers Cys797 alkylation, contributing to the easy replacement of osimertinib from EGFR by a high concentration of ATP.[138] In the absence of the T790M mutation, the L718V mutation was also detected in osimertinib-resistant NSCLC patients.[135] This mutation also causes resistance to the first-generation EGFR-TKIs, while afatinib retains its cytotoxic effect on these two EGFR mutations.[107] Because of the close location to Leu718, the Gly719 mutation may also drive resistance to osimertinib.[135] However, a clinical trial indicated that osimertinib displayed remarkable efficacy in NSCLC patients harboring the G719A mutation.[19]

Gly724 is located in a glycine-rich loop on the N lobe of the EGFR kinase domain, greatly contributing to the binding of relevant TKIs.[108] The G724S mutation shifts the glycine-rich loop into a new conformation, which is incompatible with drug binding, leading to a decreased affinity for the third-generation EGFR-TKIs.[108] In osimertinib-resistant patients, the G724S mutation has only been found in the Ex19del mutation.[139] For T790M-negative patients, the G724S mutation is sensitive to afatinib[139] while in T790M-positive patients, combined therapy of afatinib with osimertinib is suggested.

Mutations in EGFR exon 21. The L844V mutation lessens the binding of the third-generation EGFR-TKIs (WZ4002 and rociletinib) to the EGFR kinase domain, resulting in drug resistance.[109] Interaction and molecular dynamics simulation suggested that osimertinib has a high affinity to the L844V mutation.[110] Combined osimertinib with gefitinib or afatinib may surmount the drug resistance mediated by this mutation.[110]

$EGFR^{WT}$ gene amplification. Upregulation of $EGFR^{WT}$ has been reported in Ex19del mutant NSCLC patients after treatment with osimertinib.[13,140] This EGFR upregulation provoked resistance to osimertinib. In addition, upregulation of EGF also induces resistance to osimertinib.[13,140] Combination with EGFR mAbs may enhance the sensitivity of osimertinib in this case.

Deletion of T790M mutation. After receiving osimertinib treatment, deletion of T790M has been reported in T790M mutant patients.[13] Such cases have been frequently accompanied by other mechanisms of osimertinib resistance.[13] Identifying the concomitant mechanism of drug resistance contributes to the determination of the treatment strategy to overcome this chemoresistance.

Defective degradation of EGFR. $EGFR^{WT}$ is internalized into the cytoplasm after activation of the downstream signaling pathway and is degraded in lysosomes. However, clathrin-mediated internalization of mutant EGFR could not induce its degradation, resulting in a continuous activation of its downstream cascades.[111] In osimertinib-resistant cells, clathrin inhibition by pitstop initiated micropinocytosis-dependent internalization of EGFR, which prompted the degradation of mutant EGFR and inhibited activation of related downstream signaling pathways.[111] This study suggested that reactivation of degradation of mutant EGFR may be a promising strategy to overcome resistance to the third-generation EGFR-TKIs,[111] although only further investigations may confirm the clinical efficacy of clathrin inhibition in cancer with resistance to the third-generation EGFR-TKIs.

EGFR-dependent activation of PKCδ. An intriguing phenomenon whereby EGFR knockdown could reverse drug resistance, which could not be reversed by EGFR-TKIs, has been observed in resistant cells harboring a mutant EGFR.[112] A further study indicated that a new role of activating-mutant EGFR, which is independent of EGFR kinase activity, stimulates the survival of TKI-resistant NSCLC with mutant EGFR.[112] In detail, TKI-induced EGFR dimerizes with other membrane receptors of the tyrosine protein kinase family such as Anexelekto (AXL) as well as HER2.[112] It should be noted that AXL is known to serve as a cancer driver and thus has been associated with poor survival in various aggressive malignancies

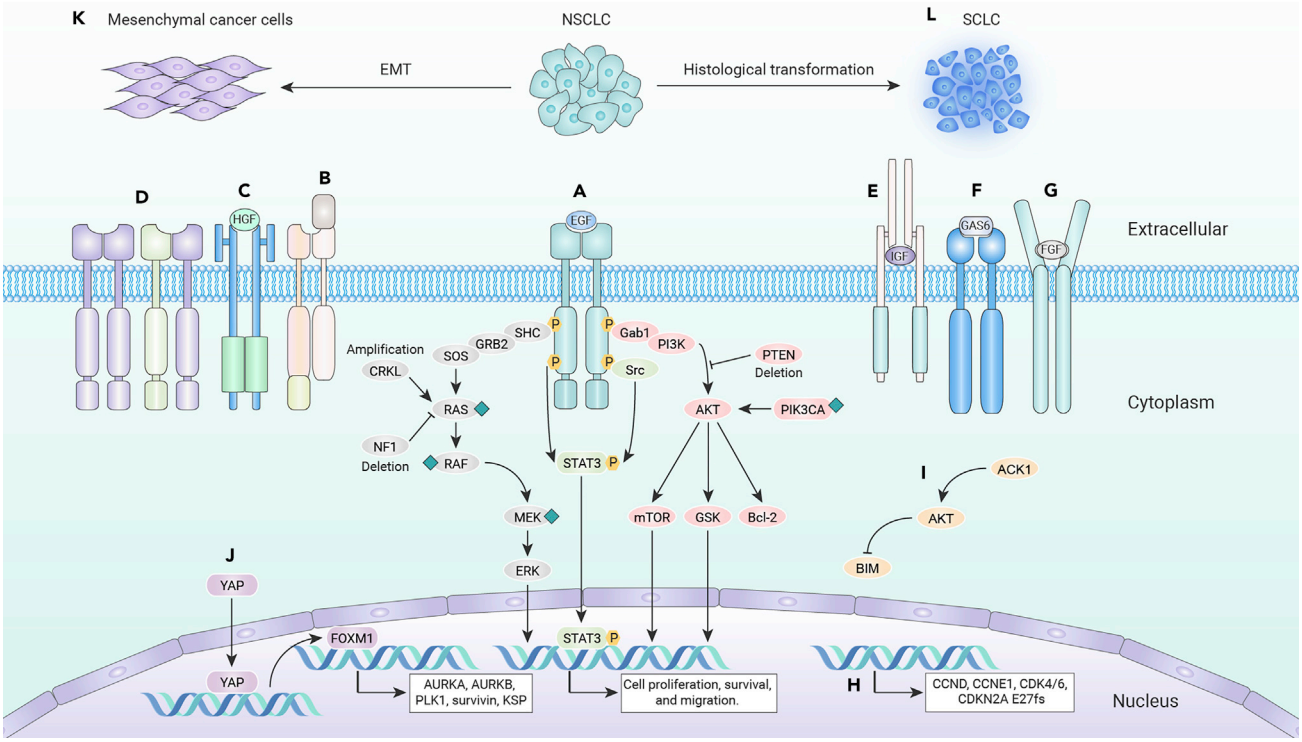

Figure 5. EGFR-independent mechanisms of resistance to third-generation EGFR-TKIs (A) Activation of downstream EGFR signaling pathways. NRAS mutation, KRAS mutation, BRAF mutation, MEK1 mutation, deletion of NF1, or amplification of the CRKL gene sustains the activation of the MAPK/ERK signaling pathway. Deletion of the PTEN or PIK3CA mutation results in constitutive activation of the PI3K/AKT signaling pathway. (B) Oncogene fusions trigger sustained activation of survival-related signaling pathways. (C–G) Amplification of tyrosine kinase receptors including MET (C), HER2/3 (D), IGF1R (E), AXL (F), and FGFR1 (G). (H) Alteration of cell-cycle-related genes. (I) Aberrant phosphorylation of ACK1 enhances the activation of the AKT signaling pathway, leading to decreased BIM levels. (J) Increased nuclear translocation of YAP upregulates the expression of FOXM1, which promotes the expression of SAC members. (K) Epithelial-mesenchymal transition (EMT). (L) Transformation from NSCLC to SCLC.

including NSCLC.[141] This heterodimer triggers phosphorylation of Tyr1173 and the activation of PLCγ2 (phospholipase Cγ2), leading to nuclear translocation of protein kinase Cδ (PKCδ) and development of resistance to third-generation EGFR-TKIs.[112] PKCδ also activates AKT and nuclear factor κB (NF-κB) signaling pathways.[112] Consistently, combined inhibition of both PKCδ and EGFR brings about robust regression of resistant NSCLC with EGFR mutations.

EGFR-independent mechanisms

Aberrant activation of downstream EGFR signaling pathways Activation of MAPK/ERK signaling pathway. Several alterations, such as NRAS mutations (G12V, G12R, Q61K, and E63K), KRAS mutations (G12S, G12A, Q61H, G12D, G13D, and A146T), BRAF mutations (G469A and V600E), MEK1 mutations, deletion of neurofibromin 1 (NF1), a GTPase-activating protein which negatively regulates the RAS/MAPK pathway, and gene amplification of v-crk sarcoma virus CT10 oncogene homolog-like (CRKL), provoke the sustained activation of RAS/RAF/MEK/ERK signaling pathway.[13,27,142,143] Combined therapy with MEK inhibitors may effectively overcome drug resistance. The recent development of KRAS inhibitors has been suggested as a new potential strategy to address this drug resistance.[144] In addition, Src homology 2 domain-containing phosphatase (SHP2) acts as the key cascade for the activation of MAPK/ERK signaling pathway.[113] Data from the NSCLC mouse model indicated that treatment with IACS-13909 an allosteric SHP2 inhibitor alone or IACS-13909 plus osimertinib markedly reduced tumor growth and prolonged survival time, suggesting the importance of further assessment of SHP2 inhibitors.[113]

Activation of PI3K/AKT signaling pathway. Deletion of phosphatase and tensin homolog (PTEN) increased phosphatidylinositol 3-kinase (PI3K) levels and induced hyperactivation of AKT, resulting in resistance to the third-generation EGFR-TKIs.[13,114] Peroxisome proliferator-activated receptor-γ (PPARγ) agonists such as rosiglitazone restore the sensitivity of PTEN-deleted cells displaying resistance to EGFR-TKIs via promotion of autophagy.[114] PIK3CA is one of the PI3K isoforms, and PIK3CA mutations abnormally activate AKT. Specifically, the PIK3CA mutations, E545K, E542K, R88Q, N345K, and E418K, have been reported to confer resistance to osimertinib.[27,142,143,145] The PIK3CA mutations E545K, E81K, and E542K were also described in rociletinib-resistant patients.[27,145]

Oncogenic fusions. Studies have found that 3%–10% of patients who received second-line treatment of osimertinib developed resistance due to oncogenic fusions, including CCDC6-RET, PCBP2-BRAF, AGK-BRAF, FGFR3-TACC3, NTRK1-TPM3, RET-ERC1, NCOA4-RET, GOPC-ROS1, ESYT2-BRAF, and EML4-ALK.[27,128,142,146–149] SPTBN1-ALK was found in the first-line treatment of osimertinib but not in second-line treatment.[128,142,143] Combination therapy greatly contributes to overcoming oncogenic fusion-dependent drug resistance to third-generation EGFR-TKIs. For instance, selective RET inhibitors, such as BLU-667 plus osimertinib, were effective in CCDC6-RET-mediated drug resistance in NSCLC.[98]

Activation of bypass survival signaling pathways. MET gene amplification. The MET signaling pathway is the most frequent bypass survival signaling pathway as an acquired resistance mechanism to EGFR-TKIs.[128,142,143,150,151] MET activated by hepatocyte growth factor potentiates tumor cell survival through persistent activation of EGFR downstream signaling pathways, boosting the emergence of resistance to EGFR-TKIs.[128,150,151] Combination of crizotinib, a multi-targeted MET inhibitor, and osimertinib, or alternatively capmatinib and afatinib, could be active in MET gene amplification-dependent resistance to osimertinib.[115,116] This combination also acted on MET exon 14 skipping-mediated resistance to osimertinib in NSCLC with mutant EGFR.[117] A phase Ib study also confirmed the efficacy of the combination therapy of savolitinib, a potent and selective MET TKI along with osimertinib in drug-resistant NSCLC.[118]

Table 3. General resistant mechanisms toward the third-generation EGFR-TKIs and corresponding countermeasures

Resistant mechanisms	Third-generation EGFR-TKIs	Countermeasures
EGFR C797S	osimertinib	first-generation EGFR-TKIs[99–101]
	rociletinib	fourth-generation EGFR-TKIs[93,102–104]
	WZ4002	
EGFR C797N/G	osimertinib	NA
EGFR L792H	osimertinib	chemotherapy (docetaxel)[105]
		cetuximab[105]
EGFR L792F/ R/Y/V/P/I/S	osimertinib	NA
EGFR G796R	osimertinib	chemotherapy (docetaxel)[105]
EGFR G796S/D	osimertinib	NA
EGFR M766Q	osimertinib	neratinib or poziotinib[106]
EGFR L798I	rociletinib	NA
EGFR E762K	osimertinib	NA
EGFR P794S	osimertinib	NA
EGFR L718Q	osimertinib	afatinib[107]
	WZ4002	
	rociletinib	
EGFR L718V	osimertinib	NA
	WZ4002	
EGFR G724S	osimertinib	second-generation EGFR-TKIs[108]
EGFR L844V	WZ4002	gefitinib[109]
	rociletinib	afatinib[109]
		osimertinib[109,110]
$EGFR^{WT}$ or EGF amplification	osimertinib	EGFR mAbs
Deletion of T790M mutation	osimertinib	according accompanied mechanism
Defective degradation of EGFR	osimertinib	clathrin inhibitors[111]
EGFR-dependent activation of PKCδ	osimertinib	PKCδ inhibitors[112]
MAPK/ERK signaling pathway	osimertinib	MEK inhibitor[13]
	rociletinib	SHP2 inhibitor[113]
	WZ4002	
PI3K/AKT signaling pathway	osimertinib	PPARγ agonist[114]
	rociletinib	
Oncogene fusions	osimertinib	RET inhibitor,[98] etc.
MET amplification	osimertinib	MET inhibitor[115–118]
	rociletinib	
	CNX-2006	
HER2/3 amplification	osimertinib	HER2/3 inhibitor (U3-1042)[119,120]
	rociletinib	
Exon16-skipping HER2 mutation	osimertinib	afatinib[121]
IGF/IGF1R amplification	osimertinib	IGF/IGF1R inhibitor[122,123]
	WZ4002	
GAS6/AXL amplification	osimertinib	AXL degrader[124]
		AXL inhibitor[124]
Alteration of cell-cycle-related genes	osimertinib	CDC4/6 inhibitor[125]
ACK1 activation	ASK120067	ACK1 inhibitor[26]
SAC components activation	osimertinib	AURKA/AURKB inhibitor[126,127]
	rociletinib	PLK1 inhibitor[126]
		Survivin inhibitor[126]
		KSP inhibitor[126]
FGF2/FGFR amplification	osimertinib	FGF2/FGFR1 inhibitor[128]
EMT	osimertinib	EMT inhibitor (JMF3086)[129]
	rociletinib	Twist1 inhibitor[129]
		ZEB1 inhibitor[127]
		YAP inhibitor[127]
SCLC transformation	osimertinib	chemotherapy (etoposide and carboplatin)[130]

HER2/3 gene amplification. Amplification of *HER2* arose in 2% or 5% cases that received first-line or second-line osimertinib treatment, respectively.[142,143] HER2 gene amplification reduced the sensitivity of T790M-mutant cells to osimertinib and rociletinib.[128] Plasma cfDNA analysis of one patient with the L858R/T790M mutation revealed that the occurrence of exon16-skipping *HER2* mutation rendered the patient resistant to osimertinib.[121] This mutation expresses HER2D16, leading to osimertinib resistance via a steroid receptor coactivator (src)-independent signaling pathway.[121] Combining osimertinib with afatinib could synergistically repress the growth of tumor cells with HER2D16.[121] HER3 forms heterodimers with other HER family members and activates the PI3K-AKT signaling pathway, which further contributes to the development of resistance to the third-generation EGFR-TKIs.[152] A triple mixture of monoclonal antibodies simultaneously targeting EGFR, HER2, and HER3 repressed the growth of T790M-expressing tumors.[119] This antibody triplet, also containing cetuximab and trastuzumab, inhibited C797S-expressing tumors. Unlike osimertinib, which induces apoptosis, this mAb triplet enhanced the degradation of the three receptors and induced cellular senescence.[119] Consistently, combined treatment with these three mAbs and subinhibitory doses of osimertinib displayed a synergistic effect and eliminated tumors.

U3-1042 is a promising HER3 inhibitor that has been recently discovered.[120] A phase II study demonstrated that the combination of U3-1042 and osimertinib could significantly ameliorate the patient prognosis.[120] Owing to the characteristics of the HER3 heterodimer, U3-1042 can substantially overcome resistance to the third-generation EGFR-TKIs caused by C797S, *MET* amplification, *BRAF* mutations, and other drug-resistance modalities.[120]

IGF/IGF1R amplification. Aberrant expression of insulin-like growth factor (IGF)/IGF1 receptor (IGF1R) triggers continuous activation of MAPK/ERK and PI3K/AKT signaling pathways, hence provoking resistance to osimertinib in cancers.[122,123] Combination with IGF1R or IGF inhibitors could restore osimertinib sensitivity.[122,123]

GAS6/AXL amplification. In lung cancer, AXL and its ligand GAS6 are upregulated after resistance to osimertinib.[13,153] Degradation of AXL is slower in osimertinib-resistant cells than in drug-sensitive cells.[124] AXL also drives intrinsic resistance to osimertinib.[154] Activated AXL by its ligand GAS6 triggers activation of MEK/ERK and PI3K/AKT pathways and boosts resistance to third-generation EGFR-TKIs.[154] Agents inducing AXL degradation (such as yuanhuadine) or inhibition (BGB324) may be a potentially effective treatment to surmount resistance to osimertinib.[124]

Alteration of cell-cycle-related genes. In 12% and 10% of NSCLC patients treated with first-line and second-line osimertinib, cell-cycle-related genes were altered, including gain of *CCND*, *CCNE1*, and *CDK4/6*, as well as *CDKN2A E27fs*.[142,143] cfDNA analysis of 41 osimertinib-treated NSCLC patients discovered that CDC4/6-positive patients had a poor response to osimertinib and shorter PFS.[155] Indeed, CDC4/6 inhibitors such as palbociclib enhanced the response to osimertinib.[125]

Activation of ACK1-related signaling pathway. A human phospho-RTK array on ASK120067-resistant lung cancer cells with the T790M mutation revealed that phosphorylation of activated cdc42-associated tyrosine kinase 1 (ACK1) was upregulated in resistant cells.[26] A further study revealed that activation of ACK1 drives resistance to ASK120067 through the AKT-BIM (Bcl-2-like protein 11) signaling pathway.[26] ACK1, a non-receptor tyrosine kinase, is a survival kinase associated with tumor cell survival.[156] Consistently, ACK1 inhibitors effectively restored the sensitivity of resistant cells to ASK120067.

Activation of spindle assembly checkpoint components. A previous study of lung cancer cells indicated that AURKA (Aurora kinase A, one of the spindle assembly checkpoint [SAC] components) activity significantly increased in both osimertinib-resistant and rociletinib-resistant cells compared with parental cells.[126] Activation of AURKA by its coactivator targeting protein for Xklp2 (TPX2), which is upregulated simultaneously, promotes mitosis and suppresses phosphorylation of BIM, hence conferring drug resistance.[126] Combined with MLN8237 (AURKA inhibitor) effectively improved the sensitivity of resistant cells to osimertinib or rociletinib, and this combination should be directed toward eliminating residual cancer cells before the acquired resistance occurs, which may prevent the development of resistance.[126] Another study of T790M-negative NSCLC demonstrated that increased nuclear translocation of Yes-associated protein (YAP) upregulated Forkhead box protein M1 (FOMX1), which triggered an increase in the abundance of SAC components, containing AURKA, Aurora kinases B (AURKB), Polo-like kinase 1 (PLK1), survivin, and kinesin spindle protein (KSP).[127] These alterations may increase resistance to EGFR-TKIs, other multiple serine/threonine kinase inhibitors, and TKIs.[127] Targeting these cascades may enhance the sensitivity of resistant cells to third-generation EGFR-TKIs.[127] In addition, loss of neurofibromin 2 (NF2), which inhibits YAP's phosphorylation and induces nuclear translocation of YAP, has also been found in osimertinib-resistant cells.[127]

Other factors. Amplification of *FGF2/FGFR*, *RB1* mutations, and *KIT* mutations, for example, may also develop resistance to third-generation EGFR-TKIs.[13,127,128] For the activated bypass signaling pathway, combined therapy would be considered as a promising strategy to overcome resistance.

Histologic transformation. Epithelial-mesenchymal transition. The epithelial-mesenchymal transition (EMT) is a paramount molecular mechanism for the escape of lung cancer from treatment with EGFR-TKIs.[157] A previous study found that osimertinib-resistant lung cancer cells displayed mesenchymal cell-like characteristics, such as loss of E-cadherin and upregulation of vimentin, indicating that EMT may also drive osimertinib resistance.[157] Another study indicated that zinc finger E-box-binding protein 1 (EZB1) and the YAP/FOXM1 axis are central regulators of EMT-associated resistance and represent therapeutic vulnerabilities in targeting this drug-resistance phenotype.[127] JMF3086, a dual histone deacetylase and HMG coenzyme A reductase inhibitor, disrupted the Src/Hakai and Hakai/E-cadherin interaction, reversing E-cadherin expression and attenuating vimentin and stemness; this resulted in restoration of gefitinib and osimertinib sensitivity in lung cancer cells.[157] Blockage of Twist1, which could induce EMT, also decreased resistance to the third-generation EGFR-TKIs.[129]

Small cell lung cancer transformation. The transformation from NSCLC to small cell lung cancer (SCLC) is another escape mechanism of a cancer cell from treatment, which has been confirmed in 14% of patients with acquired resistance to EGFR-TKIs.[158] A study reported that patients harboring this transformation also failed to respond to osimertinib.[130] The levels of both EGFR protein and mRNA were decreased in SCLC. In addition, SCLC did not display EGFR signaling pathway addiction, which may account for EGFR inhibitor resistance. The underlying mechanism of transformation remains unclear. Recent studies pointed out that SCLC transformation may be associated with the inactivation of Rb and p53.[159,160] Etoposide and carboplatin are reported to be active in two cases of SCLC transformed patients.[130]

EXPLORATION OF NEXT-GENERATION EGFR-TKIs

To overcome the EGFR mutation-mediated resistance to third-generation EGFR-TKIs, great attention have been dedicated since 2015/2016 to explore the next generation of EGFR-TKIs, known as the fourth generation (Table 4). Since the fourth generation of EGFR-TKI is an emerging area, *de novo* library screening as well as receptor structure-based drug design studies provide original hit compounds, while the evolved structure-activity relationships provide important guidelines and rationales for the discovery and iterative development of new drugs. After years of innovative research, several promising drug candidates have been evaluated in pre-clinical stages and phase I studies.[28,165–169]

EAI045

A novel EGFR allosteric inhibitor hit compound with a thiazole amide skeleton, EAI001, was discovered by a library screening comprising ~2.5 million compounds displaying special selectivity for mutant EGFR.[102] The IC_{50} value of EAI001 against L858R and T790M mutant EGFR kinase reached 24 nM, while the IC_{50} value for the $EGFR^{WT}$ was more than 50 μM.[102] EAI045 emerged from medicinal chemistry-based optimization of EAI001 (Figure 6A).[102] At a concentration of 1 mM ATP, the IC_{50} of EAI045 to T790M/L858R mutant kinase was 3 nM, hence being more than 1,000-fold selective when compared with $EGFR^{WT}$, in which the inhibition is not affected by the concentration of ATP.[102] According to the crystal structure of EAI001 binding to T790M-mutant EGFR, EAI045 may also bind to allosteric sites in the EGFR kinase domain and requires EGFR to stay at the C-helix-out conformation.[102] Molecular dynamics simulations revealed the mechanism underlying the selectivity of EAI045 toward mutant EGFR. In $EGFR^{WT}$, the Leu858 side chain is stably buried in the hydrophobic allosteric pocket and prevents the binding of EAI045 to EGFR.[170] In the L858R mutant EGFR, Arg858 induces the allosteric pocket to open, thus enabling the binding of EAI045 to EGFR.[170] Other similar mutations that could expose the allosteric pocket, such as L861Q, may also bear high affinity toward EAI045.[170] However, EAI045 exhibited poor antitumor activity and incomplete inhibition of EGFR autophosphorylation in cancer cells.[102] In $EGFR^{WT}$ asymmetric dimers, the C-lobe of the "activator" subunit turned the C-helix located at the N-lobe of the "receiver" subunit into an in conformation.[102] Hence, only the "receiver" subunit is activated in $EGFR^{WT}$. In mutant EGFR dimers, both subunits could activate the downstream signaling pathway while EAI045 only binds to one subunit, resulting in incomplete inhibition of mutant EGFR.[102] Combined with other agents that could block EGFR dimerization, they could enhance the efficacy of EAI045.[102] More precisely, cetuximab effectively enhanced the activity of EAI045 toward L858R/T790M/C797S triple FGFR mutant cells.[102]

Table 4. Progress of the next-generation EGFR-TKIs

Drug	Company	Targeted mutation	Active mechanism	Stage	Reference
EAI045	Novartis	T790M/C797S	reversible	pre-clinical	Jia et al.[102]
		L858/C797S	non-ATP competitive		
		L858R/T790M/C797S			
JBJ-04-125-02	Johnson & Johnson	T790M/C797S	reversible	pre-clinical	To et al.[104]
		L858/C797S	non-ATP competitive		
		L858R/T790M/C797S			
CH7233163	Roche Pharma	T790M/C797S	reversible	pre-clinical	Kashima et al.[161]
		L858/C797S	ATP competitive		
		Ex19del/C797S			
		Ex19del/T790M/C797S			
		L858R/T790M/C797S			
TQB3804	ChiaTai TianQing	Ex19del/T790M/C797S	NA	phase I	Liu et al.[162]
		L858R/T790M/C797S			
BBT-176	Bridge Bio	Ex19del/T790M/C797S	NA	phase I	Pharmabiz[163]
		L858R/T790M/C797S			
BLU-945	Blueprint Medicines	Ex19del/T790M/C797S	NA	pre-clinical	Schalm et al.[164]
		L858R/T790M/C797S			
Brigatinib[a]	TAKEDA Pharma	Ex19del/T790M/C797S	reversible	FDA approved	Uchibori et al.[103]
		L858R/T790M/C797S	ATP competitive		

[a]Multiple-target inhibitor.

For the T790M mutant EGFR without L858R, although a decreasing trend occurred with the increasing ATP concentration, EAI045 still displayed a promising inhibition effect on the T790M mutation.[102] However, the aforementioned molecular dynamics simulations could not explain this robust EAI045 inhibition of T790M/C797S EGFR mutation.[170] The crystal structure of EAI045-T790M/C797S/V948R mutant EGFR indicated that EAI045 could bind to a deep pocket between the ATP pocket and the C-helix, thus requiring adequate space created by the C-helix-out rotation (Figure 6B).[171] Amino acids deletion pulls the C-helix toward the ATP-binding pocket and constrains the allosteric site to a small volume, leading to the low affinity of EAI045 to the Ex19del EGFR mutation.[102] However, EAI045 is inactive as a single agent and is only active when combined with EGFR-targeted mAbs, such as cetuximab.[102] Cetuximab's on-target $EGFR^{WT}$ toxicities may limit its clinical translational potential.

JBJ-04-125-02

An iterative process of synthesizing structural analogs of EAI001 was applied, whereby a new compound, designated as JBJ-04-125-02, was obtained (Figure 6A).[104] The crystal structure revealed that, like EAI045, JBJ-04-125-02 also binds to the allosteric pockets in the C-helix-out conformation of EGFR.[104] Intriguingly, JBJ-04-125-02 binding induced a novel conformation of the A-loop that seems to be stabilized by a hydrogen bond between the piperazine group of the compound and Glu865 in the A-loop (Figure 6C).[104] JBJ-04-125-02 displayed a cytotoxic effect on L858R, L858R/T790M, or L858R/T790M/C797S mutations without coadministration of cetuximab.[104] JBJ-04-125-02 also lacks binding affinity toward $EGFR^{WT}$ or the Ex19del mutant.[104] Further research indicated that osimertinib might enhance the binding affinity of JBJ-04-125-02 to EGFR, leading to a more potent antitumor activity.[104] These data suggested that combining a covalent mutant-selective allosteric EGFR-TKI may be a useful treatment strategy for some of the lung cancer patients who are resistant to the third-generation EGFR-TKIs. However, both EAI045 and JBJ-04-125-02 cannot overcome the resistance mediated by the Ex19del/T790M/C797S triple mutant.[102,104]

CH7233163

A novel compound, CH7233163 (IC_{50} = 0.28 nM), which is capable of overcoming the resistance mediated by the Ex19del/T790M/C797S triple mutation, has been isolated from a massive chemical library (Figure 6A).[161] Compared with $EGFR^{WT}$, CH7233163 exerted a more selective inhibition on various EGFR mutants (e.g., L858R/T790M/C797S, L858R/T790M, Ex19del/T790M, Ex19del, and L858R).[161] Further tests verified the significant antitumor activity of CH7233163 in cancer cells with the Ex19del/T790M/C797S or L858R/T790M/C797S triple mutation.[161] The crystal structure of CH7233163-L858R/T790M/C797S triple mutant EGFR revealed that CH7233163 interacts with the ATP-binding pocket via hydrogen bonds and CH/π interactions but not with the Ser797 residue (Figure 6D).[161] Compared with osimertinib, CH7233163 not only binds to the P-loop and the hinge region but directly interacts with Met790 residues.[161] Compared with EAI001, CH7233163 binds to the C-helix in the conformation of EGFR, contributing to its high affinity toward Ex19del/T790M/C797S mutation.[161]

Other compounds

TQB3804 (Figure 6A) and BBT-176 are novel fourth-generation EGFR-TKIs isolated by pharmaceutical enterprises.[162,163] Pre-clinical study showed that TQB3804 and BBT-176 displayed an outstanding inhibitory effect on Ex19del/T790M/C797S and L858R/T790M/C797S triple mutation.[162,163] BLU-945 is another fourth-generation EGFR-TKI targeting the T790M/C797S mutation reported in ESCO.[164] *In vitro* data suggested that BLU-945 achieved robust inhibition of Ex19del/T790M/C797S and L858R/T790M/C797S triple mutation, rather than $EGFR^{WT}$. Cell-derived xenograft and patient-derived xenograft models consistently showed that single treatment with BLU-945 or coadministration with osimertinib/gefitinib significantly blocked the

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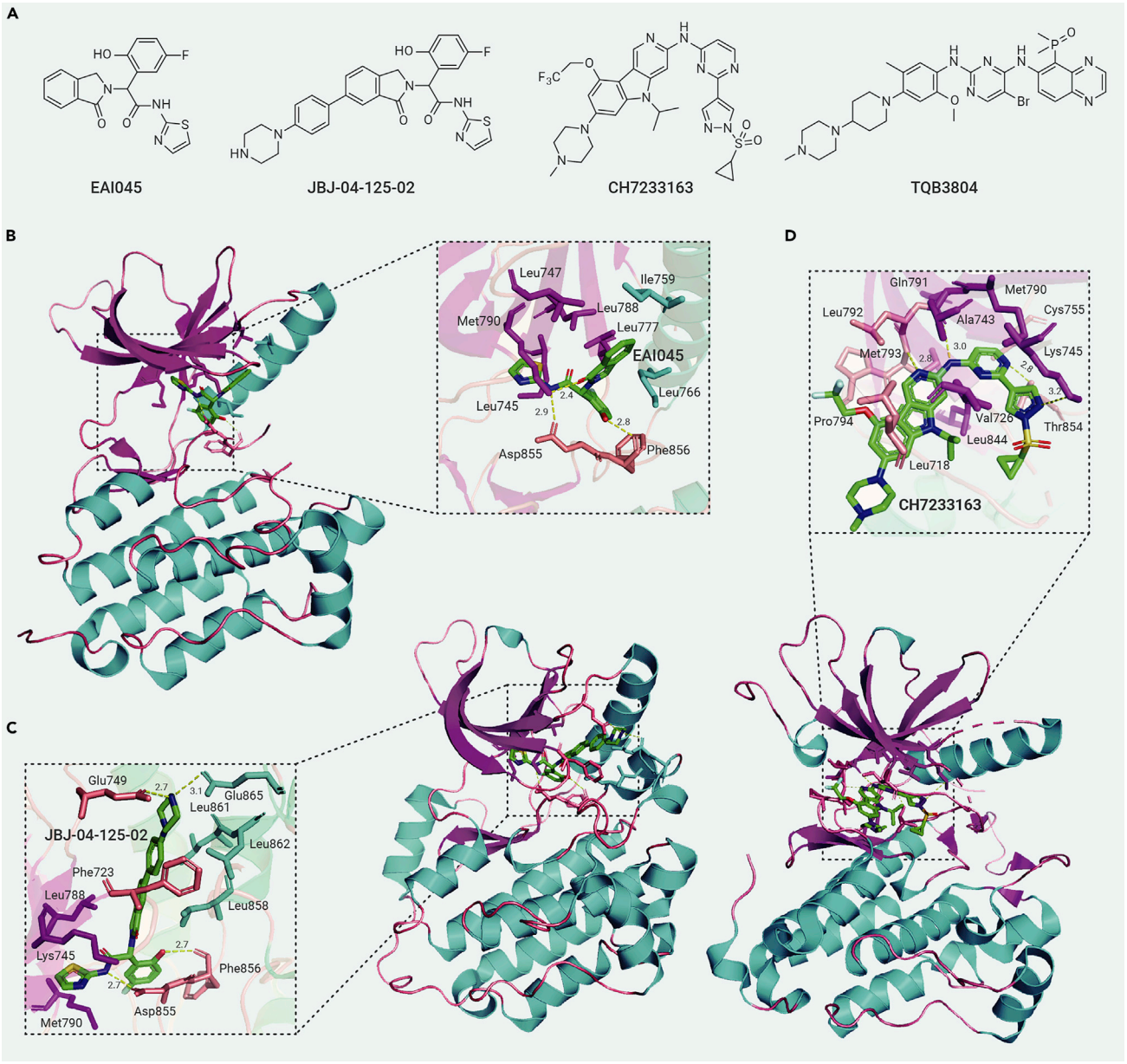

Figure 6. Crystal structure determination of the fourth-generation EGFR-TKIs (A) The published chemical structure of the fourth-generation EGFR-TKIs. (B–D) EAI045-EGFR T790M/C797S/V948R (PDB: 5ZWJ) (B), JBJ-04-125-02-EGFR T790M/V948R (PDB: 6DUK) (C), and CH7233163-EGFR L858R/T790M/C797S (PDB: 6LUB) (D) crystal structures are shown as a cartoon diagram by PyMol 2.4.1. Compounds are shown as a color-coded stick model (C, green; O, red; N, blue; S, yellow; F, pale-cyan). Distinct hydrogen bonds are shown as yellow dashed lines.

progression of tumors with the Ex19del/T790M/C797S mutation.[164] Brigatinib was identified as a next-generation inhibitor targeting anaplastic lymphoma kinase (ALK).[28] A series of screening data suggested that brigatinib potently inhibits the proliferation of cells harboring the triple EGFR mutation.[103] Moreover, several small-molecule compounds, including the 4-aminopyrazolopyrimidines, tri-substituted imidazoles, and 2-aryl-4-aminoquinazolines, have also been reported to be able to surmount C797S mutation-mediated drug resistance.[28] Additional clinical trials for these fourth-generation EGFR-TKIs may offer a promising therapeutic strategy to overcome mutant EGFR-based TKI resistance in various cancers.

SEQUENCING TECHNOLOGY ACCELERATING THE REALIZATION OF EGFR-TARGETED PRECISION THERAPY

Gene sequencing based on tumor tissue may help discover cancer driver genes and target genes for treatment, serving as an ideal "compass" for precision therapy. The development of NGS provides a powerful tool for gene sequence determination, contributing to the molecular typing of tumors and EGFR-targeted precision medicine.[172,173] Compared with traditional sequencing technologies such as fluorescence *in situ* hybridization, amplification refractory mutation system PCR (ARMS-PCR), and droplet digital PCR (ddPCR), NGS can detect known and unknown EGFR mutations, gene copy-number variation, gene rearrangement, and other gene abnormalities via large-scale and high-throughput methods.[174] Various driver genes contribute to cancer progression, and there also have been multiple mutations or comutations in different cases. Different driver genes or mutations require different targeted treatments, leading to arduous selection in the clinic. For example, NSCLC with common or uncommon EGFR mutations displays a greatly variable response to different EGFR-TKIs, for which NGS could effectively determine the precise therapeutic strategy and also predict patient prognosis.[173,175–177] For EGFR inhibitor-resistant patients,

heterogeneous and unknown drug-resistance mechanisms greatly limit the clinical application of traditional sequencing technology. In contrast, NGS can identify the mechanism underlying drug resistance and provide effective means to guide the overcoming of resistance to EGFR-TKIs;[175–177] this could facilitate the transformation of a malignant disease to a chronic disorder and help patients with advanced malignant tumors to achieve long-term survival.

In some cases it is difficult to obtain tumor tissues, such as an unresectable advanced tumor or high-risk biopsy. Liquid biopsy is a newly emerging technique and constitutes a new dawn for patients from whom tumor specimens cannot be obtained.[178] Moreover, compared with a single-lesion needle biopsy, liquid biopsy can better capture the molecular heterogeneity of different clonal populations in a given tumor.[178] ctDNA detection based on liquid biopsy has rapidly become a crucial means of standard tumor biopsy, even a potentially alternative method. Present methods for ctDNA detection comprise cobas PCR, ARMS-PCR, ddPCR, BEAMing, and NGS. The ability of cobas PCR, ARMS-PCR, ddPCR, and BEAMing to detect common EGFR mutations (Ex19del, L858R, and T790M) was assessed, whereby it was found that different methods have their own advantages in detecting different mutations.[179] These four methods could guide individualized medicine based on EGFR inhibitors by detecting known EGFR mutations. Nevertheless, these methods are deficient in their abilities to detect unknown gene alterations, making it difficult to meet the demands of precision medicine therapy. NGS detected ctDNA in 15,000 patients with advanced cancer, consisting of 37% lung cancer, 14% breast cancer, 10% colorectal cancer, and 38% other cancers, and indicated that compared with data from tissues in The Cancer Genome Atlas database, the correlation of EGFR mutations between ctDNA and tissue detection was 92%.[180] These data suggested that ctDNA could also be the biomarker for cancer and guide precision medicine targeting EGFR.[180] In fact, patients who received EGFR therapy can benefit from NGS of ctDNA.[176,181,182] In particular, EGFR mutations are detected in ctDNA by NGS, which is more instructive for EGFR-targeted therapy. However, NGS of ctDNA is prone to false negativity due to the low concentration of ctDNA, especially in early-stage tumor or low-burden tumor patients.[183] An American Society of Clinical Oncology report evaluated the guiding significance of ctDNA in urine and blood for rociletinib treatment in NSCLC. Taking histological T790M mutation detection as a standard, plasma and urine sensitivity was 80.9% and 81.1%, respectively.[184] Patients were then divided into three groups according to the type of detected samples, including tissues, plasma, and urine. Based on the results of this detection, patients in each group received rociletinib treatment. The authors found, remarkably, that there was no significant difference in ORR and mDoR among these three groups.[184] Moreover, T790M mutation-negative patients in these three distinct groups of body fluids were not overlapping,[184] indicating that detection of ctDNA from different body fluids may substantially decrease the false-negative rate. Another study showed that the detection of ctDNA in both urine and plasma could effectively identify Ex19del and L858R mutations.[185] Taken together, NGS of multiple body fluids or tissues is conducive to the realization of personalized medicine based on targeted EGFR.

CONCLUSIONS AND FUTURE PERSPECTIVES

EGFR inhibitors have enormous benefit for cancer patients. However, since tumor heterogeneity and genomic instability are hallmarks in tumor biology, the theme of anticancer drug resistance becomes inevitable for such EGFR inhibitors.[13,16,25,186–190] The present review describes that highly heterogeneous mechanisms mediate resistance of EGFR-mutated tumors to the third-generation EGFR-TKIs. Diverse and distinct mechanisms of chemoresistance emerge and coexist simultaneously. Since distinct resistance mechanisms require different treatment strategies, identification of precise molecular mechanisms of drug resistance is essential in overcoming chemoresistance. The discovery of new resistance mechanisms and selection of the proper treatment strategies is of paramount importance. The emergence of NGS and liquid biopsy provides a powerful tool for the precise surmounting of resistance to EGFR inhibitors.[175–177,179–185] Through the detection of known or unknown resistance mechanisms via dynamic monitoring achieved by applying NGS, accurate overcoming modalities can be developed in the clinic, which is conducive to the long-term survival of cancer patients.

How to improve the efficacy of EGFR inhibitors in cancer treatment is another important issue. Sequential therapy is an effective strategy to enhance the clinical benefit of patients from EGFR inhibitors. Based on the integration and analysis of clinical data from several independent clinical trials, the OS of NSCLC patients who received different sequential therapies has been predicted. It was found that sequential treatment of the first-/second-generation EGFR-TKIs, the third-generation EGFR-TKIs, and chemotherapy or sequential treatment of the third-generation EGFR-TKIs and chemotherapy may be the preferred strategy for NSCLC treatment to achieve longer OS.[25] Another study indicated that sequential treatment of afatinib and osimertinib prolonged the mPFS of NSCLC patients to 21.9 months; also, the mOS values for this sequential treatment were not reached after 4.7 years of follow-up.[186] Compared with the FLAURA study of osimertinib,[17,18] sequential treatment of afatinib and osimertinib seems to be an optimal choice to maximize OS. A phase IV study (NCT04413201) that compared osimertinib alone versus afatinib followed by osimertinib is still in the patient recruitment phase. Unfortunately, high-quality RCTs for evaluation of sequential EGFR inhibitor therapy in cancers are still overwhelmingly rare. Moreover, it is unclear whether sequential treatment of osimertinib and the fourth-generation EGFR-TKIs can be used as a more efficient strategy. Hence, high-quality clinical trials are urgently needed to assess the efficacy of different sequential EGFR inhibitor therapies.

Combination therapy such as immunotherapy is another meaningful strategy to increase the benefit of patients from EGFR inhibitors.[191] Data from NEJ026, RELAY, and ARTEMIS studies showed that combination of EGFR inhibitors with anti-angiogenic therapy significantly prolonged PFS but not OS.[192–194] A phase III study indicated that combining EGFR inhibitors with chemotherapy markedly extended PFS and OS.[195,196] Combination therapy could also effectively overcome resistance to third-generation EGFR-TKIs induced by activation of bypass signaling pathways. For instance, a combination of cetuximab, trastuzumab, and low-dose osimertinib induced degradation of EGFR and HER2 and reduced the abundance of several bypass signaling cascades such as MET, AXL, and HER3, which are able to provoke the onset of resistance to osimertinib.[197] Other combination therapies for combating resistance phenomena have also been discussed herein. Nevertheless, the combination of several drugs has also resulted in more side effects, implying that personalized medicine and timely treatment should be of much concern when using combination therapy in the clinic.

For some rare drug-resistance mechanisms it is difficult to conduct clinical trials with a sufficient number of patients, leading to arduous investigation of potential strategies to overcome drug resistance to third-generation EGFR-TKIs. Furthermore, the aberrant rate and altered form of EGFR are also greatly cancer specific, representing similar problems concerning the small sample size hence being too small to meet the needs of clinical trials for certain cancers. A master protocol is an integrated experimental scheme designed to resolve multiple issues, including an umbrella trial, basket trial, and platform trial.[198] These trials consist of a series of substudies that share key design and implementation elements, yielding a better coordination effect than when they are designed and implemented separately.[198] Therefore, it is of great clinical value to carry out a master protocol related to large-scale clinical trials to evaluate the antitumor effect of the third-generation EGFR-TKIs and customize strategies to overcome drug resistance.

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AUTHOR CONTRIBUTIONS

X.D., B.Y., and Q.A. performed data collection and analyses. X.D. and X.C. organized the review and prepared the manuscript. Y.G.A., X.C. and J.X. revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

LEAD CONTACT WEBSITE

http://shmc.fudan.edu.cn/html/xiajinglin.html