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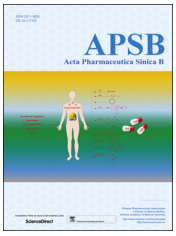

REVIEW

Mechanisms of resistance to EGFR tyrosine kinase inhibitors

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Abstract Since the discovery that non-small cell lung cancer (NSCLC) is driven by epidermal growth factor receptor (*EGFR*) mutations, the EGFR tyrosine kinase inhibitors (EGFR-TKIs, *e.g.*, gefitinib and elrotinib) have been effectively used for clinical treatment. However, patients eventually develop drug resistance. Resistance to EGFR-TKIs is inevitable due to various mechanisms, such as the secondary mutation (T790M), activation of alternative pathways (c-Met, HGF, AXL), aberrance of the downstream pathways (*K-RAS* mutations, loss of *PTEN*), impairment of the EGFR-TKIs-mediated apoptosis pathway (BCL2-like 11/*BIM* deletion polymorphism), histologic transformation, ATP binding cassette (ABC) transporter effusion, *etc.* Here we review and summarize the known resistant mechanisms to EGFR-TKIs and provide potential targets for development of new therapeutic strategies.

Abbreviations: ABC, ATP binding cassette; ABCB1, ATP binding cassette, sub-family B, member 1; ABCC1, ATP binding cassette, sub-family C, member 1; ABCC10, ATP binding cassette, sub-family C, member 10; ABCG2, ATP binding cassette, sub-family G, member 2; AKT, protein kinase B; ALK, anaplastic lymphoma kinase; AXL, Anexelekto; BCL-2, B-cell CLL/lymphoma-2; BCL2L11/BIM, BCL2-like 11; BH3, BCL2-homology domain 3; BRAF, v-RAF murine sarcoma viral oncogene homolog B1; CML, chronic myelogenous leukemia; CRKL, Crk-like protein; EGFR, epidermal growth factor receptor; EGFR-TKIs, epidermal growth factor receptor tyrosine kinase inhibitors; EGFRvIII, EGFR variant III; EML4, echinoderm microtubule-associated protein-like 4; EMT, epithelial mesenchymal transition; ERK1/2, extracellular signal-regulated kinases; FGFs, fibroblast growth factors; FGFRs, fibroblast growth factor receptors; GAS6, growth-arrest-specific protein 6; HER, human epidermal receptor; HGF, hepatocyte growth factor; IGF, insulin growth factor; IGFBPs, IGF-binding proteins; IGF-1R, IGF-1 receptor; IL, interleukin; IL-6R, IL-6 receptor; JAK, janus kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase; NSCLC, non-small cell lung cancer; PDGFs, platelet-derived growth factors; PDGFRs, platelet-derived growth factor receptors; PI3K, phosphatidylinositol-3-kinase; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase,catalytic subunit alpha; PTEN, phosphatase and tensin homolog; RAF, rapidly accelerated fibrosarcoma; RAS, rat sarcoma; RTK, tyrosine kinase receptor; SF, scatter factor; SOCS3, suppressor of cytokine signaling 3; STAT, signal transducers and activators of transcription; TKs, tyrosine kinases; TKIs, tyrosine kinase inhibitors; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

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1. EGFR signal pathway and cancers

EGFR, also known as ERBB1 and HER1, is a transmembrane tyrosine kinase receptor (RTK). EGFR is a member of the human epidermal receptor (HER) family and a crucial component of cell signal pathways. Binding with ligands (EGF and TGF-α) leads to conformational changes in EGFR and homodimerization or heterodimerization with other HER family members. There is subsequent autophosphorylation of the cytoplasmic tyrosine kinase (TK) domain with the help of adapter proteins (*e.g.*, SHC and GRB-2), which triggers downstream signaling. There are three main downstream pathways: (1) rat sarcoma (RAS)/rapidly accelerated fibrosarcoma (RAF)/mitogen-activated protein kinase (MAPK) pathway; (2) phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT) pathway and (3) janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway, which stimulates mitosis, leading to cell proliferation and inhibition of apoptosis[1]. These pathways are crucial in normal cell growth (Fig. 1).

EGFR also serves as a stimulus for cancer growth. *EGFR* gene mutations and protein overexpression, both of which activate downstream pathways, are associated with cancers, especially lung cancer. The importance of EGFR to lung cancers supports the concept of 'oncogene addiction'. Tyrosine kinase inhibitors (TKIs) have been used to treat the cancer harboring *EGFR* mutations or aberrant activation of EGFR. TKIs can inhibit the EGFR TK domain reversibly through competitive binding with ATP[2]. TKIs also lead to tumor cell death through BCL2-like 11 (BIM)-mediated apoptosis. However, patients with *EGFR*-activating mutations benefit from treatment with EGFR-TKIs (*e.g.*, gefitinb and erlotinib) for less than approximately 1 year, after which drug resistance develops.

The etiology of EGFR-TKI resistance is complex. According to the cell signal transduction pathway, the etiology of EGFR-TKI resistance can be divided into subsequent categories.

2. *EGFR* mutations induce drug resistance, especially the secondary mutation T790M

The *EGFR* gene, located in the 7p12 – 14 region in the short arm of chromosome 7, consists of 28 exons. The tyrosine kinase function is encoded by exons 18 – 24. Currently, more than 90% of the known *EGFR* mutations reside in exons 19 – 21 (Fig. 2). The rate of mutation in exon 19 is the highest, accounting for more than 60% of overall mutations[3].

2.1. *Secondary mutation—T790M*

A secondary mutation of the *EGFR* gene reported in 2005 conferred acquired resistance to EGFR-TKIs[4]. This mutation (located in exon 20) results in the substitution of methionine for threonine at position 790 (T790M) in the kinase domain. Threonine 790 has been designated as a "gatekeeper" residue, important for regulating inhibitor specificity in

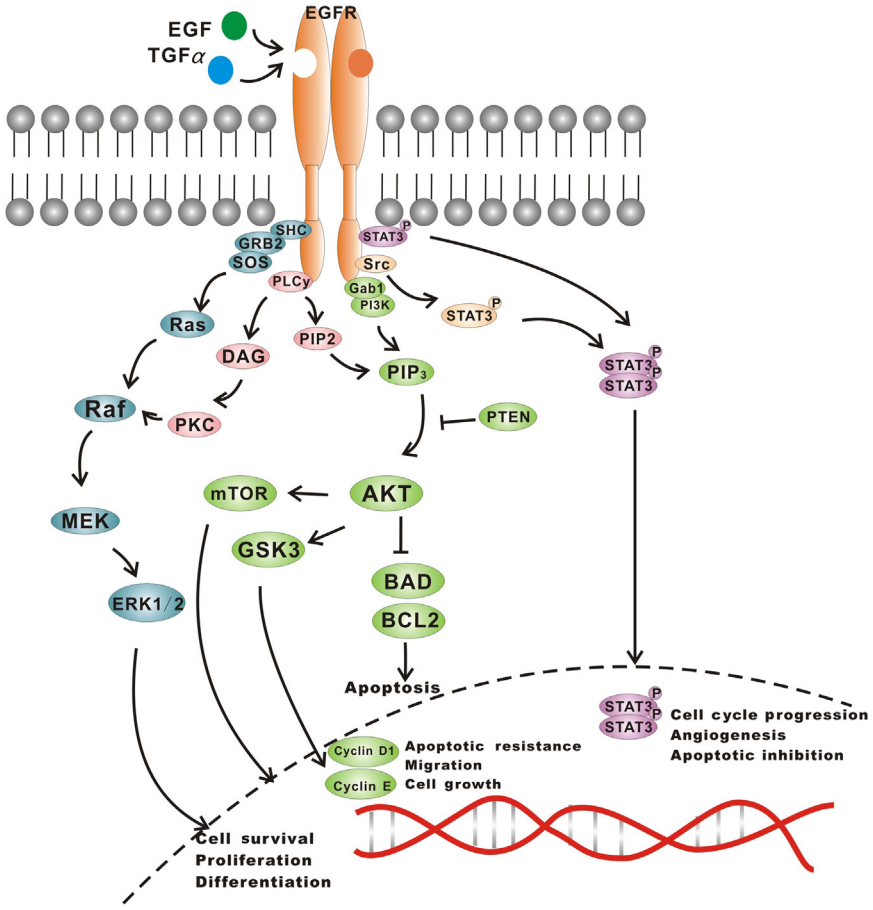

Figure 1 EGFR and its signal pathway. There is subsequent autophosphorylation of the cytoplasmic tyrosine kinase domain, which, with the aid of adapter proteins (*e.g.*, SHC and GRB-2), triggers downstream signaling. The principal pathways included: (1) RAS/RAF/MEK, (2) PI3K/AKT and (3) JAK/STAT pathways.

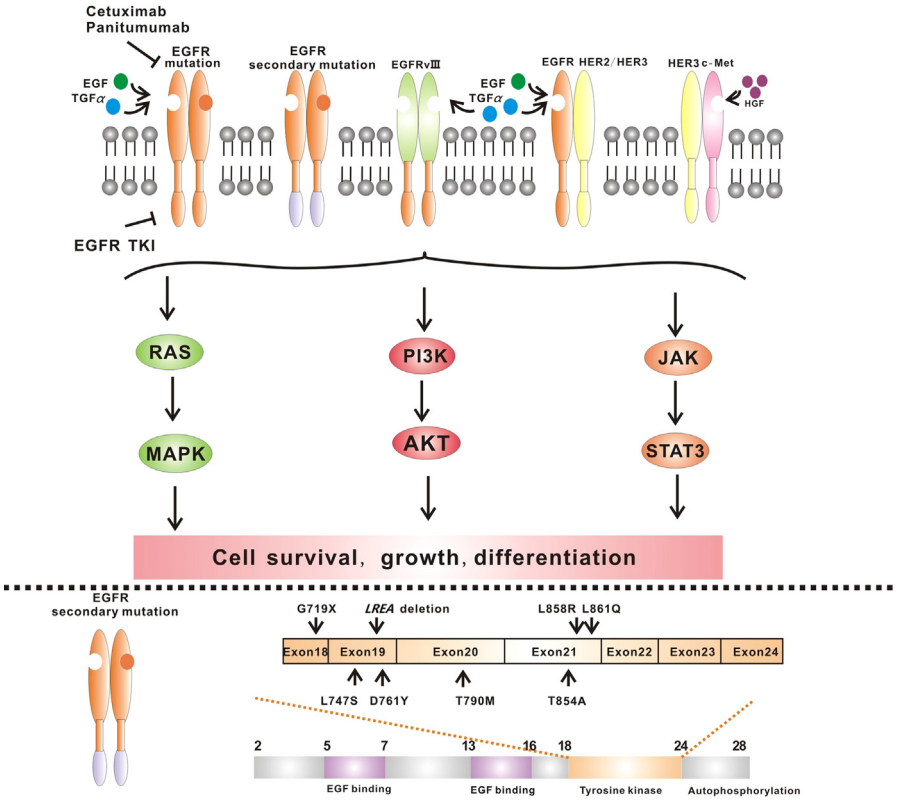

Figure 2 Aberration of HER families. Members of HER families get involved in the resistance to EGFR-TKIs. The secondary mutations of *EGFR*, *EGFR-vIII*, the overexpression of HER2 or mutations of *HER2* contribute to the resistance in the presence of EGFR-TKIs. Compared to the other HER proteins, there are currently no mutational alterations known to confer oncogenic activities to HER3. In most cases, HER3 phosphorylation is driven by one of HER family kinase partners, like HER1 and HER2. What's more, resistance can also occur through amplification of the proto-oncogene *c-Met* and the c-Met-mediated phosphorylation of HER3. HER3 serves as a key activator of downstream PI3K/AKT and MEK/MAPK signal pathways through dimerization with other HER family proteins or other molecules.

the ATP binding pocket. The T790M mutation enhances affinity of the ATP binding pocket for ATP, thus successfully competing with the TKIs, thereby conferring resistance[5]. Currently, two theories can explain the production of the second mutations: subcloning and induced mutation/acquisition[6]. Although the second mutation rarely occurs prior to treatment, it is found in approximately half of EGFR TKIs-treated patients. Experiments have identified a proportion of TKI-naive tumors that carry T790M, and these resistant clones may be selected after exposure to TKIs[7,8]. The T790M mutation can coexist with other mutations, like L858R and D761Y. The T790M mutation also possesses enhanced phosphorylating activity, especially in combination with the L858R mutation. The combination leads to lung cancer cell survival, indicating that the T790M mutant is actually an oncogene[9]. Furthermore, cyclin D1 and Hsp90 may contribute to resistance in cancer cells harboring the T790M mutant by inhibiting the degradation of EGFR and maintaining the conformation of mutant EGFR[10]. Recently, the Hsp90 inhibitor ganetespib has been shown to enhance the anti-tumor effect of TKIs[11].

2.2. *Other secondary resistance mutations: L747S, D761Y and T854A*

The non-T790M secondary mutations mainly include D761Y, L747S and T854A[12–14]. They reduce the sensitivity of mutant EGFR to EGFR-TKIs, but the resistance mechanism remains unknown. A possible explanation may be that these secondary resistance mutations modify the conformation of EGFR and the combination between EGFR and TKIs. In addition, they may affect gefitinib-induced apoptosis and inhibit BIM up-regulation. Recently, another new insertion mutation on exon 20 of *EGFR* has been reported (Pro772_His773insGlnCysPro)[15,16]. It was found in an individual who never smoked. The patient had previously been treated with cisplatin and gemcitabine, followed by carboplatin and pemetrexed. Finally, the patient developed resistance to erlotinib. Additional mutations still remain to be discovered. However, according to the reports of *EGFR*-mutated TKI-resistant patients, the frequency of non-T790M secondary mutations is low.

3. The aberrated activation of the bypass pathways induce resistance

The synchronous activation of redundant kinases also can induce resistance *via* activation of bypass pathways (Figs. 2 and 3). EGFR-TKI treatment of patients harboring such a change is not effective.

3.1. *The aberrance of other members of HER family*

The HER family is comprised of EGFR, HER2, HER3 and HER4. These receptors have a cytoplasmic TK domain which can be activated by ligand binding, followed by dimerization. Although

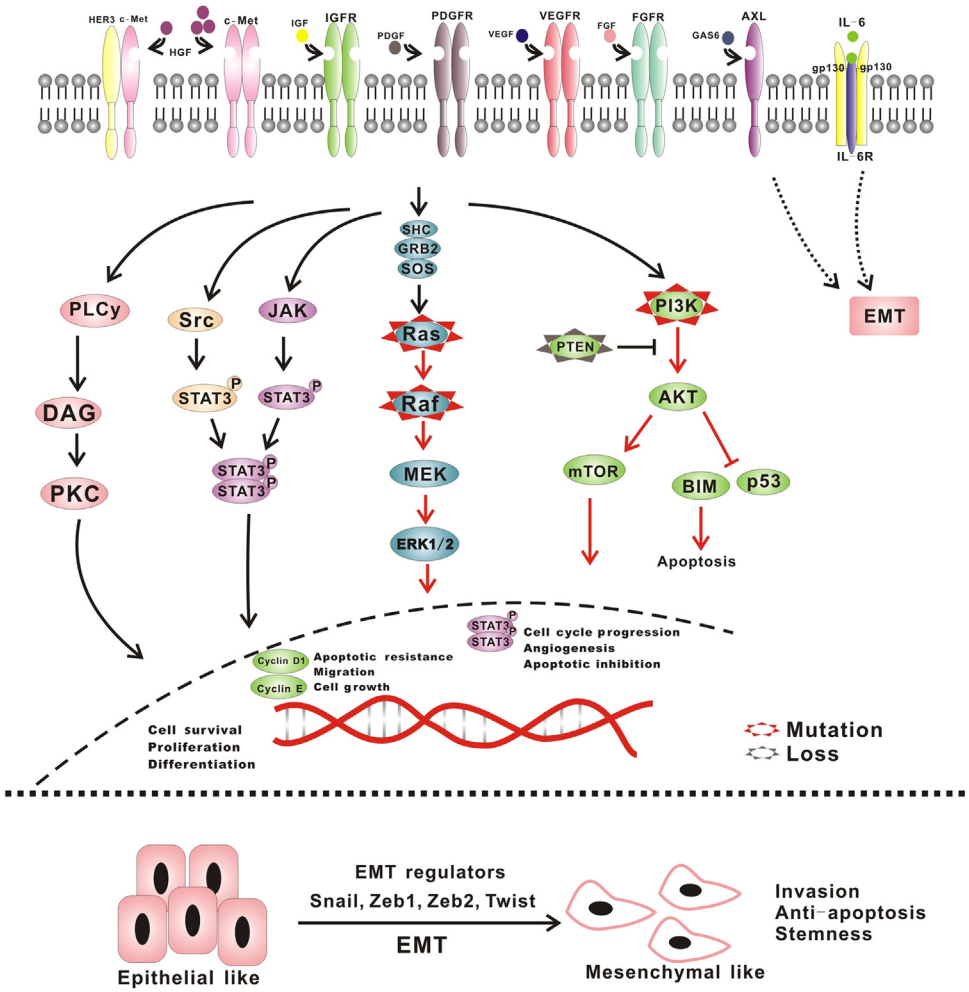

Figure 3 Synchronous activation of redundant kinases and abnormality of the downstream pathway.

HER2 appears to have no intrinsic ligand-binding capability, it can interact reversibly with ligand-activated EGFR or HER3 to form active heterodimers which activate downstream signals to govern cell proliferation and migration. Overexpression of HER2 and mutations of *HER2* are involved in resistance of EGFR-TKIs[17]. Therefore, HER2 is a useful target for treatment. A covalent, irreversible inhibitor of HER2, afatinib, can overcome the resistance of the patient harboring HER2 overexpression or *HER2* gene mutations[18]. Compared to the other HER proteins, there are currently no mutational alterations known to confer oncogenic activities to HER3, but HER3 also takes part in resistance of EGFR-TKIs. HER3 phosphorylation is driven by one of the other HER family kinase partners, like HER1 and HER2, or through amplification of the proto-oncogene *c-Met*[19]. HER3 serves as a key activator of downstream PI3K/AKT and MEK/mitogen-activated protein kinase (MAPK) pathways and contributes to survival of the most tumor cells. However, researchers have found that a heregulin–EGFR–HER3 autocrine signaling axis mediated acquired lapatinib resistance in *HER2*$^+$ breast cancer models which no longer depended on HER2–HER3–PI3K signal pathway[20]. What's more, overexpression of HER3 in COLM-5 cells can lead to significant resistance to gefitinib *in vitro* and *in vivo*[21]. All of these studies highlight the central role of HER3 in cancers. Targeting of HER3 receptor with a monoclonal antibody, such as MM-121 or MM-111 is an effective strategy currently under preclinical study and clinical evaluation[22].

3.2. *Amplification of c-Met*

c-Met is a transmembrane RTK. Binding with its ligand, hepatocyte growth factor (HGF) triggers receptor dimerization and phosphorylation, leading to conformational changes of c-Met that activates the TK domain and activates a wide range of different cellular signal pathways, including those involved in proliferation, motility, migration and invasion. Although c-Met is important in the control of tissue homeostasis under normal physiological conditions, it has also been found to be aberrantly activated in human cancers *via* gene mutation, amplification or protein overexpression. In 2007, the second most common primary resistance to EGFR-TKIs involved amplification of the *c-Met* oncogene in HCC827 NSCLC cells after exposure to gefitinib. In c-Met-resistant patients, *c-Met* amplified clones existed prior to EGFR-TKIs therapy and were selected out by treatment[23]. The resistant mechanism of EGFR-TKIs may involve HER3-PI3K/AKT signaling by maintaining HER3 phosphorylation in the presence of gefitinib, which is independent of EGFR kinase activity[19]. Besides, a high *MET* gene copy number leads to shorter survival

in patients with NSCLC. Clinical trials have demonstrated that concurrent inhibition of EGFR and c-Met can overcome resistance of EGFR-TKIs and improve patient outcomes.

3.3. *Overexpression of HGF*

HGF is the ligand for c-Met. HGF acts as a pleiotropic factor and cytokine, promoting cell proliferation, survival, motility, scattering, differentiation and morphogenesis.

Overexpression of HGF is another EGFR-TKIs resistance mechanism and it may be more common among patients with mutations who had no response[24]. One study showed that HGF induces resistance by activating c-Met which restores phosphorylation of downstream MAPK/extracellular signal-regulated kinases (ERK1/2) and PI3K/AKT pathways[25]. Interestingly, although amplification of *c-Met* activates downstream pathways by activating ERBB3, HGF induces downstream pathways through c-Met; this activation is independent of ERBB3 or EGFR. The resistance induced by HGF not only appears in the NSCLS, but also exists in breast cancers. Blockade of EGFR and the downstream pathways can overcome HGF-mediated resistance.

3.4. *The abnormality of insulin growth factor receptor (IGFR)*

As early as 2002, it had been suggested that IGF-1 receptor (IGF-1R) signaling through PI3K may represent a novel and potentially important mechanism of resistance to anti-EGFR therapy. In 2008, researchers found that the loss of expression of IGF-binding proteins (IGFBPs) in tumor cells treated with EGFR-TKIs increased the activation of IGF-IR signaling which, in turn, mediates resistance of EGFR-TKIs[26,27]. Inhibition of IGF-IR signaling disrupted the association of IRS-1 with PI3K and restored the ability of gefitinib to down-regulate PI3K/AKT signaling and to inhibit cell growth. Concomitant inhibition of both EGFR and IGFIR was required to abort PI3K signaling, and treatment of the resistant cells with an IGFIR inhibitor restored their sensitivity to EGFR TKIs. Another study found that activation of IGF-1R can alter phosphorylation state and subcellular localization of p27, which can promote cell proliferation and cell motility[28]. Thus, IGF-1R and p27 can be used to be a biomarker of cell cycle arrest and response to therapy.

3.5. *The abnormality molecules of multiple angiogenic pathways*

Angiogenesis is an essential step in tumor growth and metastasis. Impaired vascularity and hypoxia can lead to increased metastasis and treatment resistance. Thus, targeting multiple angiogenic pathways may not only improve antitumor activity but also reduce the risk of resistance[29]. Important targets for the development of novel antiangiogenic therapies include vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs), and their receptors.

3.5.1. *The VEGFs and their receptors*

The VEGF family and receptors are important regulators of angiogenesis and vascular permeability. Overexpression of vascular endothelial growth factor receptor (VEGFR) 1 in tumor cells leads to cell survival and invasion. It also reduces the inhibition by EGFR inhibitors and reduces sensitivity to gefitinib. Activation of the EGFR signal can increase the expression of VEGF. Researchers have demonstrated that a VEGF/VEGFR2 feed-forward loop in NSCLC cells expressing VEGFR2, which leads to a signal amplification and a boost in VEGF secretion, is required for establishment of fully angiogenic tumors *in vivo*. This VEGF/VEGFR2 signaling cascade *via* VEGFR2/PI3K/mTOR induces an mTOR-dependent regulation of VEGF secretion[30]. And VEGF secretion is induced by the upregulation of HIF-1α. Thus, the VEGF/VEGFR system is associated with resistance of anti-EGFR drugs through activation of downstream signal pathways *via* EGFR-independent mechanisms[31]. The therapeutic use of agents able to inhibit both EGFR and VEGFR may help to efficiently inhibit the activation of bypass pathways and overcome EGFR inhibitor resistance.

3.5.2. *The fibroblast growth factors and their receptors*

The FGFs and the receptors (FGFRs) are involved in multiple cellular functions. During embryonic development, FGFs play a part in morphogenesis. In adults, FGFs are involved in wound healing and tissue repair as well as regulating the nervous system. FGFs also participate in tumor angiogenesis. FGFR autocrine signaling has been implicated in NSCLC cell lines. In 2010, researchers found EGFR-TKIs (gefitinib) increased the expression of FGFR2 and FGFR3. Importantly, FGFR2 and FGFR3 are capable of mediating FGF2 and FGF7 stimulated ERK activation, leading to cell survival and invasion and reducing the sensitivity to EGFR-TKIs[32]. FGFR activation is an escape mechanism in human lung cancer cells resistance to afatiniab, that may compensate for the loss of EGFR-driven signaling pathway[33]. Treatment of NSCLC patients with combinations of EGFR and FGFR specific inhibitors (*e.g.*, PD173074) or FGF antibodies may be potential strategy to enhance efficacy of single EGFR-TKIs.

3.5.3. *The platelet-derived growth factors and their receptors (PDGFRs)*

PDGFs are isolated from human platelets and promote angiogenesis. As angiogenesis factors, PDGFs/PDGFRs are closely related to tumor development. PDGFs/PDGFRs can induce tumor cell proliferation and migration, and inhibit apoptosis. In 2013, Akhavan et al.[34] found that a physiologic RTK switch to the PDGFRβ was required to maintain the growth of EGFR variant III (EGFRvIII)/EGFR-activated in response to EGFR-TKIs. Combination of EGFR and PDGFR inhibitors may overcome resistance of EGFR-TKIs.

3.6. *EGFRvIII*

EGFRvIII is a tumor-specific mutation that results from in-frame deletion of 801 base pairs spanning exons 2–7 of the coding sequence. This deletion removes 267 amino acids from the extracellular domain, creating a junction site between exons 1 and 8 and a new glycine residue. EGERvIII has expressed in many kinds of tumors.

EGFRvIII confers enhanced tumorigenicity through multiple mechanisms and pathways. EGFRvIII expression is associated with the activation of downstream PI3K/AKT/mTOR pathway and increases proliferation and cell cycle progression mediated by a decrease in the level of $p27^{KIP1}$. EGFRvIII also has been shown to activate the NF-κB pathway and regulate expression of IL-8[35]. Furthermore, EGFRvIII induces angiopoietin-like 4 expression through the ERK/c-Myc pathway and promotes tumor angiogenesis in malignant gliomas[36]. Cells harboring EGFRvIII have an enhanced capacity for dysregulated growth, survival, invasion and angiogenesis. EGFRvIII is an attractive

target and predictor in cancer immunotherapy because it is not expressed in normal tissue.

3.7. Overexpression of or overactivated Anexelekto (AXL)

AXL belongs to the Tyro/Axl/Mer (TAM) family of RTK. Growth-arrest-specific protein 6 (GAS6) is a ligand for AXL. Activation of AXL increases cell migration, aggregation and growth through multiple downstream pathways. AXL was over-expressed in *EGFR*-mutant NSCLC tumor xenografts with acquired resistance to erlotinib[37]. The researchers suggested that the AXL upregulation may activate AKT, MAPK or NF-κB signaling to promote resistance to erlotinib, perhaps in association with epithelial mesenchymal transition (EMT) process *via* inducing Slug expression[38]. Inhibitors of AXL (MP-470 or XL-880) enhance erlotinib sensitivity particularly in the context of NSCLC with acquired resistance to EGFR-targeting TKIs. Therefore, the inhibitors of AXL or GAS6 may overcome the AXL-mediated resistance of EGFR-TKIs.

3.8. Excess secretion of interleukin-6 (IL-6)

IL-6 is a cytokine which plays an important role in many chronic inflammatory diseases. Binding to the membrane-bound IL-6 receptor (IL-6R) causes the recruitment of two gp130 co-receptor molecules and the activation of intracellular signaling cascades *via* gp130. However, some reports suggest that IL-6 expression is involved in the regulation of tumor growth and metastatic spread, including lung cancers. One study demonstrated that activation of the IL-6R/JAK1/STAT3 pathway induced *de novo* resistance to irreversible EGFR-TKIs in NSCLC harboring T790M[39]. Afatinib activated IL-6R/JAK1/STAT3 signaling *via* autocrine IL-6 production[40]. Inhibition of the IL-6R/JAK1/STAT3 signal pathway can reverse the resistance. JAK1 activates STAT3 activity which is stopped rapidly by the major negative regulator suppressor of cytokine signaling 3 (SOCS3) in normal cells. However, association of the IL-6 receptor with the EGFR can activate STAT3 in presence of the inhibition of SOCS3 to JAK1 and JAK2[41]. Besides, paracrine or autocrine stimulation of the TGF-β axis also increases the secretion of IL-6 and promotes resistance[42].

3.9. Amplification of Crk-like protein (CRKL)

CRKL is a member of adapter proteins that participates in signal transduction in response to both extracellular and intracellular stimuli, such as growth factors, cytokines and the oncogenic BCR-ABL fusion protein. Oncogenic CRKL activates the SOS1/RAS/RAF/ERK and SRC/C3G/RAP1 pathways. Amplification of the *CRKL* gene was observed in 1/11 lung cancer patients with *EGFR* mutations who acquired resistance to EGFR-TKIs[43]. Amplification of *CRKL* in *EGFR*-mutant cells induces resistance to gefitinib by activating ERK and AKT signaling[44]. What's more, overexpression of CRKL promotes cell invasion *via* upregulating MMP9 expression and activating ERK pathway[45]. Therefore, CRKL can be a potential target in the treatment of NSCLC.

3.10. Overexpression and activation of integrin beta1

Beta1 subunit of integrin is an adhesion molecule, sharing common downstream signaling elements with EGFR, such as the PI3K/AKT and ERK1/2 pathways. Overexpression of integrin β1 induces gefitinib resistance, accompanied by increases in cell adhesion and migration[46]. Subsequently, researchers found that β1 integrin activated an alternative survival pathway in breast cancer cells harboring resistance to lafatinib, which led to activation of β1 integrin's downstream kinases, FAK and SRC. Inhibition of β1 integrin by AIIB2 can enhance the sensitivity to EGFR-TKIs[47]. Further study found that ligand-dependent activation of integrin β1 could induce EGFR-TKIs resistance through activating c-Met and downstream pathways[48]. Kanda et al.[49] identified the integrin β1/Src/AKT signal pathway as a key mediator of acquired resistance to EGFR-targeted anticancer drugs. Researchers have also found that integrin mediates a stem-like phenotype and confers resistance to EGFR-targeted therapy through enhancing downstream coupling to a KRAS/RalB/NF-κB pathway[50]. Therefore, integrin β1 and the pathway molecules provide potential agents for overcoming the resistance mediated by integrin β1.

4. Abnormal downstream pathways induce drug resistance

The abnormal downstream pathways also can result in the resistance to EGFR-TKIs, even though the cells have not harbored any other mutations. The mutation of *K-RAS*, the loss of phosphatase and tensin homolog (*PTEN*), the mutations of *PIK3CA* and *BRAF* are the main points (Fig. 3).

4.1. K-RAS mutation

The RAS proteins include K-RAS, N-RAS and H-RAS. The RAS proteins are GTPases that are molecular switches for a variety of critical cellular activities and their function is tightly and temporally regulated in normal cells. Oncogenic mutations of *RAS* genes, which create constitutively-active RAS proteins, can result in uncontrolled proliferation or survival in tumor cells. In 2005, Pao et al.[51] showed that mutations in *K-RAS* are associated with primary resistance to single-agent gefitinib or erlotinib. If the mutations occur at codons 12, 13, 59, 61, 63, 116, 117, 119 or 146, its structure is altered by binding sites for guanine, affecting normal function. The effects of these mutations can be translated either in a reduction of the activity of oncoprotein GTPases, blocking it into the active form bound to GTP, or in decreased binding affinity and increasing the change in GDP by GTP attachment[52]. In total, 80% of *K-RAS* mutations occur in codon 12, and other mutations are mainly located in codons 13 and 61. *RAS* gene alteration is poor prognostic factors for survival of patients with NSCLC. Additionally, several studies have clearly demonstrated that RAS uses additional effectors to promote tumorigenesis[53]. Very little is known about the relevant mechanisms.

4.2. Loss of PTEN

PTEN dephosphorylates PI-(3, 4, 5)-triphosphate, which mediates activation of AKT, thereby negatively regulating the PI3K/AKT/mTOR pathway and leading to G1 cell cycle arrest and apoptosis. In addition, PTEN inhibits cell migration and spreading through regulation of focal adhesion kinase as well as regulates p53 protein levels and activity.

PTEN deleted on chromosome 10 is a tumor suppressor gene on chromosome 10q23.3 and encodes a 403 amino acid dual-specificity lipid and protein phosphatase. The loss of *PTEN* has only been investigated in a small number of NSCLC cases. However, *PTEN* loss contributes to erlotinib resistance in *EGFR* mutant lung cancer by activation of AKT and EGFR[54,55]. The

absence of PTEN protein expression is an independent prognostic marker in early stage resected lung adenocarcinoma. Inhibition of the PI3K/AKT/mTOR signal pathway can be an effective strategy to NSCLC harboring the *EGFR* activating mutations that acquires resistance to both TKIs and radiotherapy due to *PTEN* loss[56].

4.3. Mutations of BRAF (v-RAF murine sarcoma viral oncogene homolog B1)

BRAF, another component of the EGFR/RAS/RAF signal transduction pathway, encodes a RAS-regulated kinase that mediates cell growth, differentiation, apoptosis and malignant transformation. Mutations of *BRAF* (G469A, V600E and V599E) were found in several tumors, including malignant melanoma and colorectal cancer. Thus, *BRAF* mutations also induce drug resistance[57]. Activating *BRAF* mutations, especially the common mutant V599E, induces constitutive activation of the signal transduction pathway, providing a potent pro-mitogenic force that drives malignant transformation. The *BRAF* V599E mutant shows greatly increased activity in the RAF/MEK/ERK pathway both *in vitro* and *in vivo*. Here, BRAF provides a new target for the treatment to overcome the mutant *BRAF*-mediated resistance.

4.4. A downstream mutation in PIK3CA

The *PIK3CA* gene, encoding a catalytic subunit of the PI3K, is mutated or amplified in various neoplasias, including lung cancer. Infrequently, a downstream mutation in *PIK3CA* has been identified as a mechanism of resistance[58]. Recently, a study demonstrated that *PIK3CA* mutations frequently coexist with *EGFR* or *K-RAS* mutations[59]. Patients with single *PIK3CA* mutation in NSCLC have poor prognosis. The role of mutant *PIK3CA* in oncogenic signaling requires further investigation, including development of novel targets for therapeutic intervention in cancers harboring *PIK3CA* mutations.

4.5. The aberrant expression of NF1

Neurofibromin, the RAS GTPase-activating protein, is encoded by *NF1* gene. A recent study demonstrated that reduced expression of NF1 was associated with erlotinib resistance due to a failure to fully inhibit RAS/RAF/MEK/ERK pathway[60]. Combination therapy with EGFR and MEK inhibitors may restore sensitivity to EGFR-TKIs.

5. Impairment of a pathway that is essential for EGFR-TKIs-mediated apoptosis: a common intrinsic deletion polymorphism in the gene encoding BIM

BIM is a pro-apoptotic member of the B-cell CLL/lymphoma-2 (BCL-2) family, and plays a critical role in inducing cell apoptosis and tumor metastasis. Consequently, BIM has become the focus of intense interest as a potential target for cancer chemotherapy. Its upregulation is required for apoptosis induced by EGFR and EGFR-TKIs in tumors harboring *EGFR* mutations. The polymorphism switched *BIM* splicing from exon 4 to exon 3, which resulted in expression of BIM isoforms lacking the pro-apoptotic BCL2-homology domain 3 (BH3)[61] (Fig. 4). Although the polymorphism was sufficient to confer intrinsic TKI resistance in

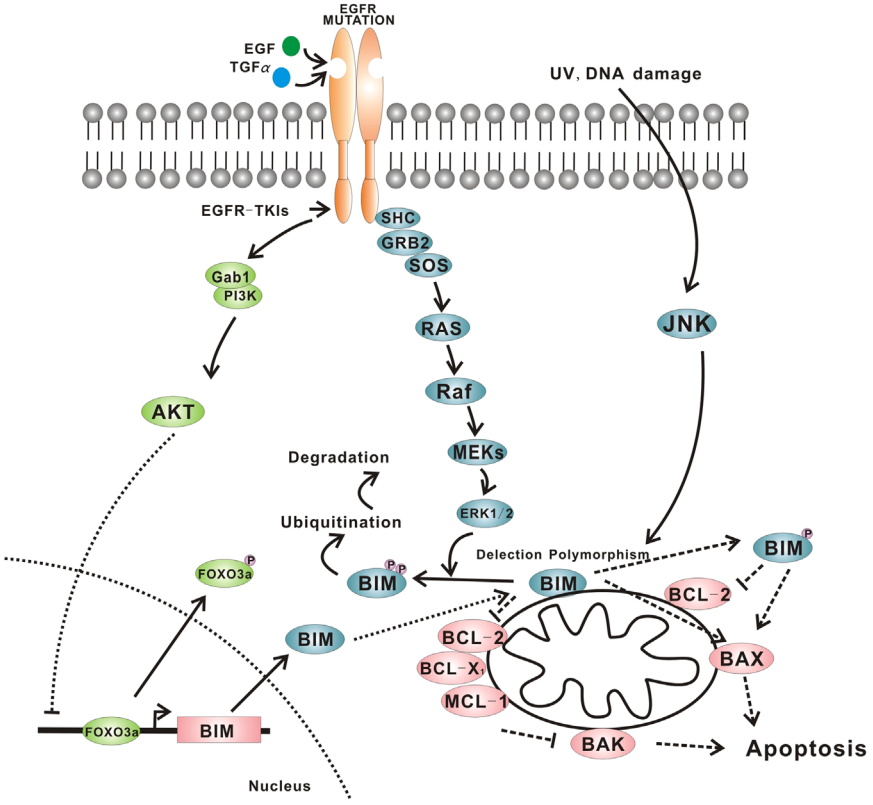

Figure 4 Apoptosis pathway mediated by BIM.

chronic myelogenous leukemia (CML) and EGFR NSCLC cell lines, this resistance could be overcome with BH3-mimetic drugs. Recently, researchers have shown that the PP2A activator FTY720 could induce apoptosis of CML cells *via* dual activation of BIM and BID and overcome various types of resistance of TKIs[62].

6. Histologic transformation

6.1. The EMT

EMT is a physiological process during embryogenesis that appears to be reinstated in adult tissues undergoing wound healing and tissue regeneration, or under certain pathological conditions, such as fibrosis and cancer. EMT is characterized by the combined loss of epithelial cell junction proteins, such as E-cadherin, and the gain of mesenchymal markers, such as vimentin and N-cadherin[63]. In the EMT process, epithelial cells lose their features, gain properties of mesenchymal, and become motile and invasive (Fig. 3).

In 2005, a transition to a mesenchymal phenotype was noted among patients treated with EGFR-TKIs. EMT may be induced by the activation of AXL *via* the PI3K/AKT pathway. One study also showed that loss of E-cadherin can activate EGFR–MEK/ERK/ZEB1/MMP2 axis, which is responsible for promoting invasion in NSCLC[64]. Tumor cells undergoing EMT are also known to increase the secretion of specific factors, including cytokines, chemokines and growth factors, which could play an important role in tumor progression. Well-established signals include those initiated by TGF-β, FGF, EGF and HGF, all of which have been shown to promote EMT in various tumor cell models. Other possible pathways or factors that have been reported to be associated with EMT include IL-8, IL-6, Notch-1, SOX9, FoxO4, SRC and CRIPTO-1[65–69]. Co-targeting the relative molecules of these pathways and EGFR can reverse the resistance mediated by EMT[70]. However, the specific mechanism of EMT still remains unknown.

6.2. Small cell transformation

Conversion to small-cell carcinoma has been seen at the time of development of resistance. Tumor cells retained the original *EGFR* mutations but developed a histopathologic small-cell phenotype, which may benefit from a standard chemotherapy for small cell lung cancer[71]. Interestingly, after a period of conventional cytotoxic treatment, susceptibility to TKIs may redevelop. Research into this phenomenon is still insufficient.

7. ATP binding cassette (ABC) effusion

The ABC transporters are transmembrane proteins that involved in transporting biologically important substrates across the cellular membranes, such as amino acids, cholesterol, hydrophobic drugs and antibiotic. Overexpression of ABC transporters can reduce drug uptake, increase drug efflux and lead to low drug density in the cytoplasm, which will come to a lower drug efficacy and finally acquire drug resistance. When EGFR-TKIs (lapatinib)

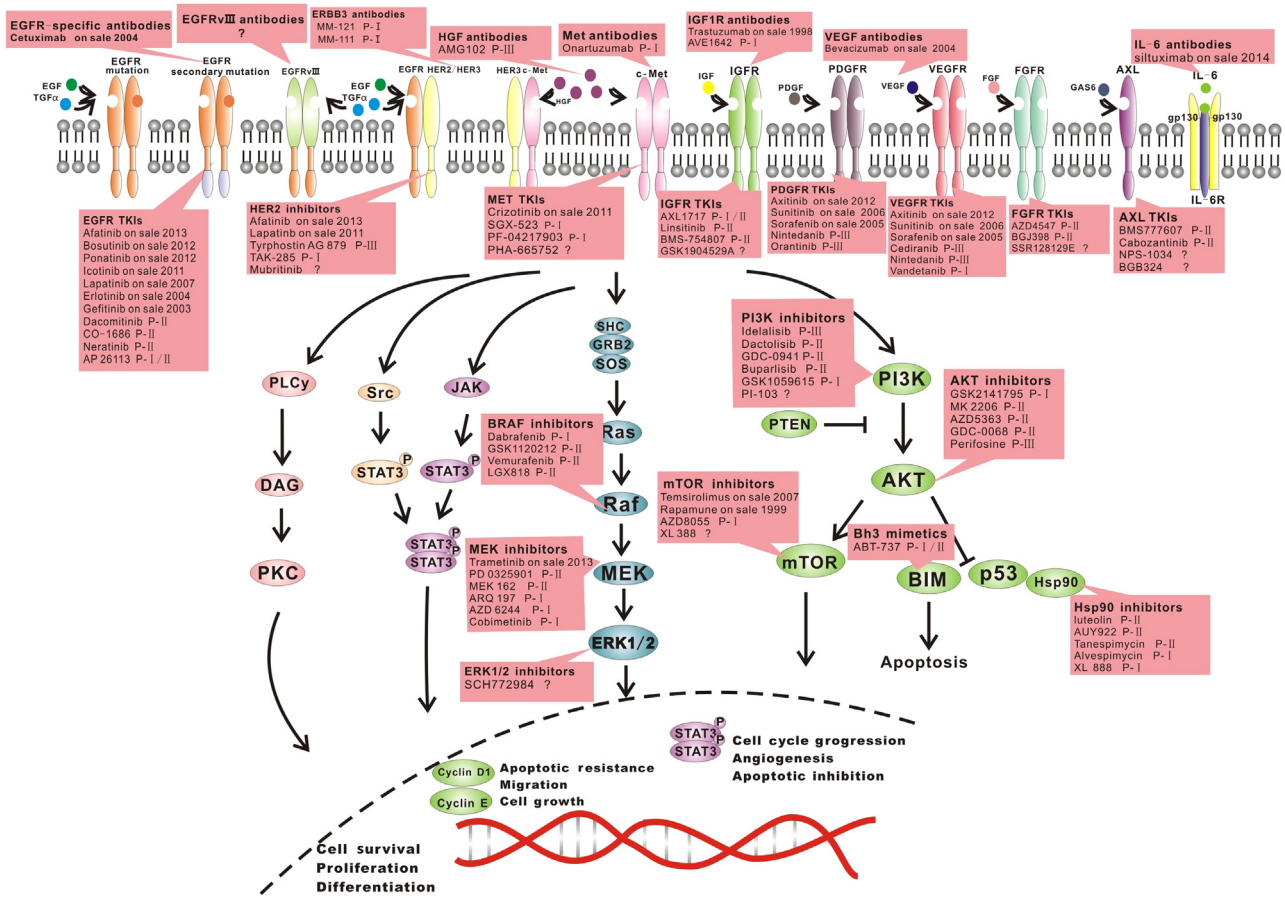

Figure 5 The potential targets for the relative treatment to overcome the resistance to EGFR-TKIs.

binds to the ATP binding cassette, sub-family B, member 1 (ABCB1) and ATP binding cassette, sub-family G, member 2 (ABCG2), substrate-binding sites with high affinity induced overexpression of ABC transporters[72]. Other ABC transporters, such as ATP binding cassette, sub-family C, member 1 (ABCC1) and ABCC10 also participate in drug resistance. Given the ABC transporter influence on TKI actions, ABC transporter inhibitors may reverse the resistance. In addition, researchers[73] found that GW583340 and GW2974, EGFR and HER-2 inhibitors can reverse ABCG2- and ABCB1-mediated drug resistance.

8. Echinoderm microtubule-associated protein-like 4-the anaplastic lymphoma kinase (*EML4-ALK*) fusion gene and the *ALK* secondary mutation

The *EML4-ALK* fusion oncogene was identified as a novel genetic alteration in NSCLC[74]. Patients harboring *ALK* rearrangements tend to be non-smokers or light smokers, have a history of adenocarcinoma, and tend to be younger in age[75]. The *EML4-ALK* fusion gene was present at a high frequency in Chinese NSCLC patients, particularly in those with adenocarcinomas lacking *EGFR/K-RAS* mutations[76]. Recently, crizotinib was identified as a potent inhibitor of ALK and MET tyrosine kinases[77]. Crizotinib was well tolerated and resulted in important tumor shrinkage in NSCLC *EML4-ALK* positive patients. However, some patients have resistance to crizotinib and other EML4-ALK inhibitors are in development. Researchers[78,79] found that the naive NSCLC patients with *ALK* rearrangements also had concurrent *EGFR* activating mutations. The resistance mechanisms to ALK TKIs may be mediated by both *ALK* secondary mutation and a bypass signaling pathway such as EGFR. These mechanisms can occur independently, or in the same cancer. Combination therapy with EGFR-TKIs and ALK inhibitors can improve the anti-tumor effect.

Table 1 Therapeutic strategies and clinical trials to overcome resistance of EGFR-TKIs, "–" stands for no drugs for therapy.

Resistant mechanism	Strategy	Clinical research	Ref.
***EGFR* mutation**			
T790M	EGFR-TKIs combined/+antibodies	Afatinib+cexitumab	80
	T790M-specific inhibitors	CO-1686/AZD9291	81,82
	c-Met inhibitors+PI3K inhibitors	GDC0973+GDC0941	83
	HSP90 inhibitors	Luteolin/ganetespib	11,84
	EGFR-TKIs+MEK inhibitors	Afatinib+ARQ 197	85
	Glycolysis inhibition+EGFR-TKIs	Afatinib+AUY922	86
Bypass pathway			
HER family abnormality	HER inhibitors+EGFR-TKIs	Afatinib/dacomitinib	18,87
c-Met amplification	EGFR-TKIs+c-Met inhibitors	Erlotinib+crizotinib Dacomitinib+crizotinib	88
HGF overexpression	EGFR-TKIs+PI3K inhibitors	Gefitinib+PI-103	89
	Triple inhibition of EGFR/Met/VEGF	–	90
IGFR abnormality	IGFR inhibitors+EGFR-TKIs	AG1024+gefitinib	–
EGFRvIII	EGFRvIII antibodies	–	–
VEGF/VEGFR abnormality	EGFR-TKIs+VEGF inhibitors	ZD6474	91
	MEK inhibitors+VEGF inhibitors	ZD6474+PD0325901	30
PDGF/PDGFR abnormality	EGFR-TKIs+PDGF inhibitors	–	–
FGF/FGFR abnormality	EGFR-TKIs+FGF inhibitors	–	–
IL-6 abnormality	IL-6 antibodies	Siltuximab	92
AXL abnormality	AXL inhibitors	NPS-1034	93
CRKL amplification	Unknown	Unknown	–
Integrin beta1 overexpression	Unknown	Unknown	–
Downstream pathway			
K-RAS mutations	PI3K inhibitors+MEK inhibitors	GDC-0941+AZD6244	94
BRAF mutations	BRAF inhibitors+MEK inhibitors	Dabrafenib+trametinib	95
Loss of *PTEN*	mTOR inhibitors/AKT inhibitors	–	–
PIK3CA mutation	EGFR-TKIs+PI3K inhibitors	Gefitinib+BKM120	96
Low expression of NF1	Unknown	Unknown	–
Apoptosis pathway			
BIM BH3 deletion	EGFR-TKIs+PP2A activator	Erlotinib+FTY720 gefitinib+FTY720	62
Histologic transformation			
EMT	EGFR-TKIs+MEK1/2 inhibitors	–	–
SCLC transformation	Platinum+VP16/EGFR-TKIs	–	–
ABC effusion	EGFR-TKIs+HER-2 inhibitors	GW583340/GW2974	73
Unknown mechanism	EGFR-TKIs combined	Afatinib+cexitumab	80
	EGFR-TKIs+glycolysis inhibitors	Erlotinib+AUY922	

9. Therapeutic strategies to overcome resistance

The existence of various resistance mechanisms is clear, and it is wise to identify the specific mechanisms in a patient so that a suitable, effective strategy can be chosen. The next generation of EGFR-TKIs and specific antibodies of the relative molecules are under development. MicroRNAs will soon be employed in treating resistance to EGFR-TKIs in cancers. Clinical trials are engaged in examining the potential targets less susceptible to resistance. The relative clinical trials or therapeutic strategies are listed in the table (Table 1[11,18,30,62,73,80–96] and Fig. 5). In practice, combined treatments or therapies with multiple targets may show more powerful efficacy, and the multi-targeting drugs may show superior efficacy.

10. Conclusions

Increasing evidence shows that the primary or acquired resistance to first- or second-generation EGFR-TKIs explains why patients who initially benefited from these treatments later do not. Though some of the mechanisms of resistance have been identified, much additional information is needed to understand and overcome resistance to these agents.

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