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Review Article

Mechanistic insights into acquired drug resistance in epidermal growth factor receptor mutation-targeted lung cancer therapy

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Oncogenic mutation of epidermal growth factor receptor kinase domain is strongly associated with clinical response to tyrosine kinase inhibitors in non-small-cell lung carcinoma. Despite an initial encouraging response, patients eventually develop drug resistance and relapse. Great efforts have been made to identify the molecular mechanisms of drug resistance. With the recognition of cancer as a whole complex system, here it is proposed that cancer may evolve drug resistance in a cancer-cell-autonomous manner as well as a non-cancer-cell-autonomous manner. The former mainly arises at three levels: the robustness of the epidermal growth factor receptor signaling network; cancer epigenetic changes; or cancer genetic alteration, which may be dependent on the therapeutics methods and treatment duration. As cancer stroma plays an essential role in lung cancerigenesis, we further discuss the potential mechanisms for drug resistance development in a non-cancer-cell-autonomous manner, which may arise from the interaction between cancer cells and cancer stroma, including stromal cells and extracellular matrix. (*Cancer Sci* 2010; 101: 1933–1938)

Lung cancer, pathologically divided into small-cell lung carcinoma and non-small cell lung carcinoma (NSCLC), is the leading cause of cancer-related death worldwide.[1] Three main sub-types of NSCLC, squamous cell lung carcinoma, adenocarcinoma, and large cell lung carcinoma, are grouped together because their prognosis and management are similar. The EGFR signal pathway, essential for normal epithelial cell proliferation and survival,[2] is frequently deregulated in lung adenocarcinoma and squamous cell carcinoma. For example, *EGFRvIII* mutation with a partial deletion of the extracellular domain encoded by exons 2–7 exists exclusively in lung squamous cell carcinoma.[3] In contrast, *EGFR* kinase domain mutation is observed in 30–60% of lung adenocarcinomas with features similar to that of bronchioloalveolar carcinoma that arises in the distal bronchioles or alveoli that initially show a specific non-invasive growth pattern,[4–6] sometimes in adenosquamous carcinoma, but seldom in pure squamous or large cell carcinoma.[7] *EGFR* kinase domain mutation is also preferentially observed in females, non-smokers, and those of East Asian ethnicity.[8] Interestingly, the gender bias of EGFR kinase domain mutation is largely due to the high prevalence of tobacco smoking in men in East Asia.[9–11]

Tyrosine kinase inhibitors, including gefitinib and erlotinib, have been developed to target EGFR in lung cancer treatment. The *EGFR* kinase domain mutation is strongly associated with clinical response to TKIs.[4–6] Although multiple molecular testing methods, including immunohistochemistry, FISH, and mutational analyses, were used to assess the EGFR status, *EGFR* mutation remains one of the most important determinants for the prediction of clinical response and survival benefit.[12] This association may be explained by the different biological functions of wild-type or mutated EGFR in contributing to cancer maintenance.[13] From the biological standpoint, EGFR-targeted therapy in those NSCLC patients with EGFR-activating mutations is targeting the key oncogene that is directly involved in tumorigenesis and is essential for tumor maintenance, thus frequently correlated with effective clinical outcome.[13]

Despite impressive and durable clinical responses, those patients with *EGFR* mutant NSCLCs almost invariably develop drug resistance after approximately 1 year of TKI treatment.[14] In the past several years, studies have revealed certain molecular mechanisms that contribute to drug resistance, including secondary *EGFR* mutation T790M and tyrosine protein kinase *MET* amplification.[15–20] With the recognition of cancer as a whole complex system, here we propose that cancer may evolve TKI resistance in a cancer-cell-autonomous manner as well as a non-cancer-cell-autonomous manner. We further discuss the cancer-cell-autonomous mechanisms from the robustness of the EGFR signaling network, to epigenetic changes, to genetic alterations in cancer cells, and the potential mechanisms for drug resistance in a non-cancer-cell-autonomous manner, which may arise from the interaction between cancer cells and cancer stroma (Fig. 1).

Drug Resistance Developed in a Cancer-Cell-Autonomous Manner

Lung cancer is a robust system featured with extreme complexity and high heterogeneity. The EGFR pathway is located at the center of converging signals for cell proliferation, motility, and other cancer cell behaviors.[2] Stress or perturbation may modulate multiple signaling pathways and trigger epigenetic changes. Genetic alteration frequently occurs during lung cancerigenesis due to genomic instability. Although these three factors might contribute to drug resistance development at different timepoints during cancer treatment, they work together to confer cancer cells with the ability to maintain stable function against drug treatment. It will be discussed how the drug resistance develops during EGFR-targeted therapy in a cancer-cell-autonomous manner.

Epidermal growth factor receptor signaling network in drug resistance development. The EGFR signal pathway contributes to multiple biological events, including normal cell proliferation, differentiation, and survival, and is frequently deregulated

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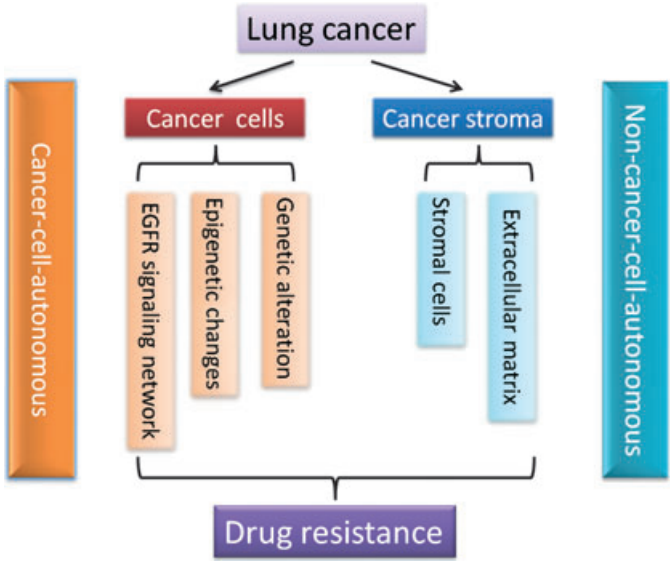

Fig. 1. Schematic illustration of the potential molecular mechanisms of drug resistance development in epidermal growth factor receptor (EGFR) mutation-targeted lung cancer therapy in a cancer-cell-autonomous and non-cancer-cell-autonomous manner. The robustness of the EGFR signaling network, epigenetic changes, and genetic alterations in cancer cells contribute to drug resistance in a cancer-cell-autonomous manner. The interaction between cancer cells and cancer stroma, including stromal cells and ECM, contributes to drug resistance in a non-cancer-cell-autonomous manner.

under pathological conditions such as in NSCLC.[2,21] Epidermal growth factor receptor belongs to the ERBB family, which consists of four closely related receptor tyrosine kinases: HER1/ERBB1; HER2/NEU/ERBB2; HER3/ERBB3; and HER4/ERBB4. After ligand stimulation, EGFR is activated through homodimerization or heterodimerization and transmits signals to downstream substrates such as PI3K/AKT, RAS/RAF/MAPK, and STAT3/5 pathways, which contributes significantly to cell proliferation and cell survival (Fig. 2).

Several EGFR downstream substrates have been indicated to be responsible for drug resistance despite the current lack of clinical evidence. Activation of the EGFR downstream signal PI3K/AKT pathway is essential for cancer cell survival (Fig. 2). An effective TKI treatment in NSCLC cell lines usually inhibits PI3K/AKT pathway activation.[14,22] Conversely, the PI3K/AKT pathway remains activated in most models of acquired drug resistance.[19,23–25] Oncogenic mutations in the PI3K catalytic subunit alpha (*PIK3CA*) have been found in 1–5% of NSCLC samples.[26,27] In contrast to other oncogenic drivers such as *KRAS*, *BRAF*, or *HER2* mutations, the *PIK3CA* mutations is not mutually exclusive with *EGFR* kinase domain mutations.[26,27] Thus, the coexistence of *PIK3CA* mutation may confer TKI resistance in those cases of NSCLC with *EGFR* mutations. This was confirmed by an *in vitro* study.[24] In breast cancer, the presence of *PIK3CA* mutations predicts a lack of response of *HER2* amplified tumors to trastuzumab treatment.[28] Similar findings have been reported in colon cancer.[29] Loss of phosphatase and tensin homolog (PTEN), which activates AKT, may confer a TKI resistance during NSCLC and colon cancer treatment.[28–30] In addition, increased internalization of ligand-activated EGFR and altered EGFR trafficking was previously found to confer drug resistance through unknown mechanisms.[31] Further investigation using lung cancer specimens may improve our understanding about whether and/or how the EGFR downstream signaling, including the PI3K/AKT pathway, contributes to drug resistance development.

The cross-talk of EGFR signaling with other receptor partners is of potential interest in the development of TKI resistance. Multiple membrane proteins including G-protein coupled receptors (GPCR), IGF-1R, and MET cross-talk with EGFR or other members of the EGFR family, such as HER2 and ERBB3.[32] For example, GPCR activation results in G-protein-mediated activation of ADAM family members, which shed pro-HB-EGF (Fig. 2). The increase in the HB-EGF amount in turn enhances EGFR signaling.[33–35] The IGF-1R protein plays an important role in tumor formation and progression through its multiple biological functions including cell proliferation, apoptosis, and angiogenesis.[36] Both *in vitro* and *in vivo* studies have indicated that the cross-talk between EGFR and IGF-1R contributes to drug resistance in EGFR-targeted therapy.[37] Multiple levels of interaction between EGFR and IGF-1R have been proposed, either through a direct association between these two receptors or through common interaction partners such as GPCR or downstream signaling molecules[38] (Fig. 2). A recent study has shown that simultaneous targeting of EGFR and IGF-1R significantly decreased the possibility of TKI resistance development in PC9 cells, an NSCLC cell line which has *EGFR* kinase domain mutation and is initially sensitive to TKI treatment.[39]

The importance of MET in contributing to TKI resistance was initially documented by Engelman *et al.*[19] In approximately 20% of patients with acquired TKI resistance, lung cancer cells display *MET* gene amplification.[19,20] Interestingly, a recent study has identified the existence of subpopulations of lung cancer cells with MET amplification prior to TKI treatment that may be responsible for drug resistance development.[40] In addition, autocrine hepatocyte growth factor production, which activates MET, also induces drug resistance.[40,41] MET contributes to EGFR TKI resistance through transactivation of ERBB3.[19] Interestingly, the PI3K/AKT pathway is the main downstream pathway activated by MET through ERBB3[19] (Fig. 2), which allows the cancer cells to maintain survival signaling even in the presence of EGFR inhibitors. Interestingly, MET-resistant cancer cells can shift back the survival-dependent oncogene to EGFR,[42] highlighting the importance of the cross-talk between MET and ERBB3. Concomitant inhibition of both EGFR and MET is very important for overcoming TKI resistance and may provide a survival benefit for NSCLC patients. Interestingly, this is somehow supported by recently released phase II trial data showing that ARQ 197, a MET inhibitor, when used in combination with erlotinib, showed a 66% improvement in median progression-free survival in patients with advanced, refractory NSCLC.

Epigenetic changes in drug resistance development. Epigenetic changes, referring to changes of the cell ''epigenome'' without the direct involvement of DNA sequence alteration, not only contribute significantly to cancer development and progression but also provide cancer cells with another means to escape from various targeted therapy. In response to various perturbations, the epigenome may change correspondingly and turn on or off genes or pathways that may confer survival benefit. Similar to genetic alterations, epigenetic changes can be transmitted to the next generation at least for a certain period. DNA cytosine methylation, gene imprinting, and chromatin remodeling are key events involved in epigenetic changes. Inhibitors towards DNA methylation and histone deacetylase (HDAC) have been developed as two main classes of drugs over past years. Here the main focus is on the potential role of HDACs in drug resistance development.

Histone deacetylases play an important role in cellular proliferation and survival.[43] HDACs actively regulate gene expression through chromatin modification in the opposing action of histone acetyltransferases (HATs) in normal cells.[44] Histone acetyltransferases transfer acetyl groups to the aminoterminal lysine residues in histones, which causes local chromatin

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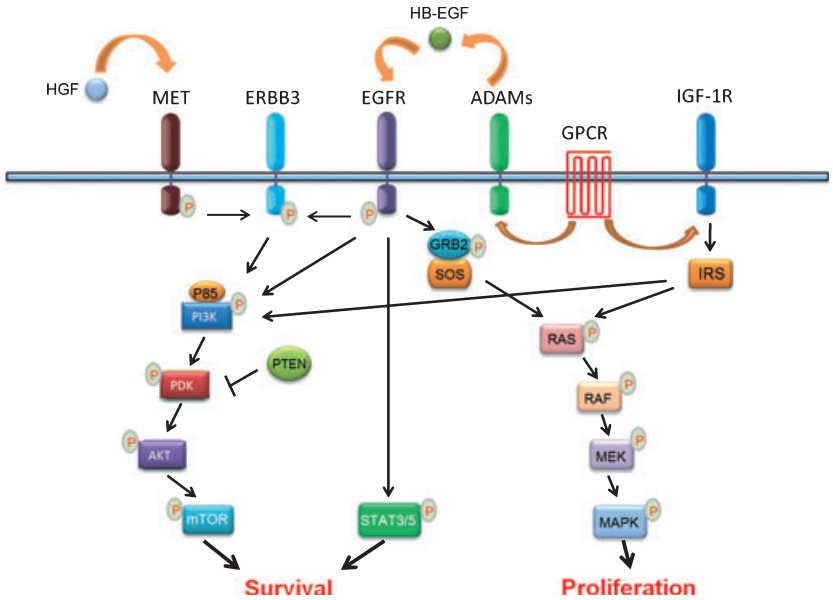

Fig. 2. Simplified schematic illustration of the epidermal growth factor receptor (EGFR) signaling network. After ligand stimulation, EGFR is activated and directly transmits signals to downstream pathways, including the phosphatidylinositol-3-kinase (PI3K)/3-phosphoinositide-dependent protein kinase (PDK)/v-akt murine thymoma viral oncogene homolog (AKT)/mammalian target of rapamycin (mTOR) and signal transducer and activator of transcription 3/5 (STAT3/5) pathways for cell survival, and the rat sarcoma viral oncogene homolog (RAS)/v-raf-1 murine leukemia viral oncogene homolog (RAF)/MAPK/ERK kinase (MEK)/MAPK pathway for cell proliferation. Phosphatase and tensin homolog (PTEN) inhibits AKT activity through PDK. The EGFR pathway cross-talks with other signaling pathways: either hepatocyte growth factor receptor (MET) activation by hepatocyte growth factor (HGF) stimulation or EGFR activation is able to transmit signals through erythroblastic leukemia viral (v-erb-b) oncogene homolog 3 (ERBB3) and results in the activation of the PI3K/AKT pathway; and G-protein coupled receptor (GPCR) activation enhances EGFR signaling through a disintegrin and metalloproteinases (ADAMs) that shed pro-heparin-binding EGF-like growth factor (HB-EGF) and increase the amount of HB-EGF. As well as the cross-talk with GPCR signaling, insulin-like growth factor-1 receptor (IGF-1R) signaling through insulin receptor substrate (IRS) activates the PI3K/PDK/AKT/mTOR and RAS/RAF/MEK/MAPK pathways, which are commonly shared by EGFR signaling. GRB2, growth factor receptor-bound 2; P, phosphorylation; SOS, son of sevenless homolog; P85, phosphatidylinositol 3-kinase regulatory subunit.

expansion and allows proteins to access and activate gene transcription.[44] Conversely, HDACs catalyze the removal of acetyl groups, which results in chromatin condensation and the repression of gene transcription.[45] The well-balanced action of HATs and HDACs in normal cells is frequently disrupted during the cancerigenesis process, which therefore confers tumor cells with the ability to efficiently silence tumor suppressor genes and genes related to cell cycle arrest, differentiation, and apoptosis.[46] In addition, HDACs also modify several non-histone proteins such as TP53, E2F1, HIF-1α, and HSP90.[47] The inhibition of HDAC activity reactivates silenced genes and induces growth arrest and apoptosis in numerous tumor cell lines.[48] Several HDAC inhibitors, including vorinostat, LBH-589, PDX-101, and MS-275, have been developed for NSCLC treatment. Interestingly, treatment of LBH-589 in lung cancer cells with EGFR mutations proves to be effective in triggering apoptosis, mainly through HSP90 acetylation and downregulation of EGFR and other key survival signaling proteins.[49] Inhibition of HDAC activity by LBH-589 also synergizes with erlotinib for treatment of lung cancer cell lines.[49]

Interestingly, an essential role of HDACs in a ''reversible drug resistance'' has been recently proposed.[39] While modeling the acute response of PC9 cells to TKI treatment, Sharma and coworkers consistently detected a small subpopulation of reversibly ''drug-tolerant'' cells, which was significantly associated with an altered chromatin state.[39] Both HDACs and the histone demethylase RBP2/KDM5A/Jarid1A are involved in TKI resistance.[39] Interestingly, continuous propagation for approximately 30 passages resensitizes these drug-tolerant cells to TKI, further supporting the view that the epigenetic alteration is dynamically involved in drug resistance and may be inheritable, at least for a certain period.[39]

Genetic alteration in drug-resistance development. Genomic instability remains one of most important obstacles for lung cancer therapy. Genetic alterations in lung cancer, especially oncogenic driver mutations of EGFR, KRAS, BRAF, HER2, and ALK fusion,[50,51] provide the driving force for cancer development and progression. Yet, it also works as the basis for the development of molecular targeted strategies in the clinic. At the same time, the genomic instability behind these essential genetic alterations enables cancer cells to evolve and find ways to escape molecular targeting.

Despite the initial encouraging response to EGFR TKI in certain lung cancer patients, genetic alteration such as *MET* amplification, found in approximately 20% of relapsed patients, sustains the normal function of cancer cells through cross-talk with the EGFR pathways. Studies have revealed that approximately half of these relapsed patients have a secondary EGFR mutation, the substitution of a threonine for methionine at position 790 (T790M).[15–18] The EGFR T790M mutation occurs at the ''gatekeeper'' residue in tyrosine kinases and confers a drug resistance analogous to BCR-ABL and KIT in imatinib-resistant CML and gastrointestinal stromal tumors, respectively.[52,53]

In contrast to primary *EGFR* mutations such as L858R and exon 19 LREA deletion mutations, *EGFR* T790M mutation cannot be inhibited by either gefitinib or erlotinib treatment in *in vitro* studies, and maintains downstream PIK3/AKT activation.[16,31,54] This has been further confirmed by *in vivo* animal model studies.[55,56] Ectopic expression of the EGFR T790M mutant is sufficient to render EGFR mutant cancer cell lines resistant to gefitinib.[24] When NSCLC cell lines such as PC-9 and H3255, which have the highly sensitive EGFR mutant, were exposed to gefitinib for a long period, they eventually acquired drug resistance and a *T790M* mutation.[23,24] Interestingly, a

rather small percentage of the EGFR alleles within one single TKI-resistant cell are observed to harbor T790M mutation in H3255 cell drug resistant model, which is termed ''allelic dilution''.[24]

In contrast to the reversible TKIs like gefitinib and erlotinib, the second generation EGFR inhibitors, the irreversible TKIs such as CL387,785, EKB-569, PF00299804, BIBW2992, and HKI-272, seem to effectively inhibit EGFR T790M and block the growth of NSCLC cell lines harboring T790M mutations.[16,24,31,54,57]

Preclinical work in mice suggests that irreversible EGFR inhibitors such as HKI-272 might not be potent enough to completely block EGFR T790M signaling *in vivo*, and the combinational therapy with inhibitors blocking downstream signaling, such as rapamycin, improves efficacy.[55] Interestingly, a novel EGFR TKI, WZ4002, efficiently inhibits EGFR phosphorylation and induces significant tumor regression in murine models of EGFR T790M.[58] Additionally, HSP90 inhibitors may effectively target EGFR mutants for degradation and thus overcome the T790M mutation.[59] As T790M remains as a good target, a better designed drug or combinational therapeutic strategies are necessary to overcome drug resistance.

Drug Resistance Development in a Non-Cancer-Cell-Autonomous Way

The complexity of cancer exists not only in cancer cells but also in tumor stroma, which is composed of stromal cells such as fibroblasts, inflammatory cells, and endothelial cells and extracellular matrix (ECM). The interplay between cancer cells and the microenvironment strongly impacts upon the cancer development and progression.[60–62] Cancer-associated infiltrated inflammatory cells or ECM has been shown to contribute to tumor progression.[60–63] Additionally, blood vessels provide cancer cells with oxygen and nutrition. Targeting both the cancer cells and the new blood vessel genesis, so called ''angiogenesis'', are under development now. The use of dual EGFR/vascular endothelial growth factor inhibitors, which target both EGFR and vascular endothelial growth factor receptor signaling pathways, may be more effective than the inhibition of one single pathway and help to overcome resistance to EGFR inhibition.[64]

Additionally, genetic alterations in tumor stroma may contribute to tumor formation. For example, the genetic inactivation of Pten in stromal fibroblasts of mouse mammary glands accelerated the initiation, progression, and malignant transformation of mammary epithelial tumours.[65] Similarly, LKB1 loss in mesenchymal cells has also been shown to cause the formation of gastrointestinal polyps.[66] The interaction between stromal cells with Scribble loss and RAS mutant tumor cells results in JNK pathway activation and cancer metastasis into distant organs.[67] Although the above mechanisms have not been identified in clinic specimens yet, genetic analyses have revealed that TP53 mutations occur in non-neoplastic stromal cells.[68] Targeting the cancer cells alone without concomitant treatment towards stromal cells in this case may be ineffective in blocking drug resistance development. A comprehensive understanding of the interaction between cancer cells and cancer stroma may provide the basis for development of better therapeutic strategies for lung cancer treatment and overcoming drug resistance.

Conclusion and Future Directions

Cancers have integrated several essential hallmarks, including uncontrolled growth, limitless proliferation, insensitivity to antigrowth signaling, sustained angiogenesis, and metastasis.[69] Cancer robustness is achieved through the interplay between cancer cells and the microenvironment, which provides cancer the ability to maintain normal function against various perturbations and to eventually develop drug resistance[70,71] The view

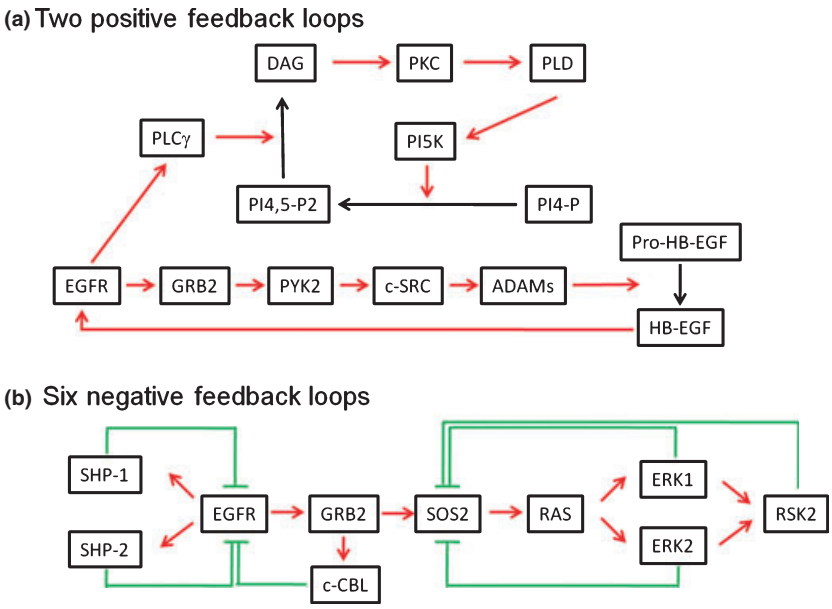

Fig. 3. Positive and negative feedback loops in the epidermal growth factor receptor (EGFR) signaling pathway. (a) Two positive feedback loops. First, the activation of proline-rich tyrosine kinase 2 (PYK2)/v-src sarcoma viral oncogene homolog (c-Src) activates a disintegrin and metalloproteinases (ADAMs) which shed pro-heparin-binding EGF-like growth factor (HB-EGF) and results in an increase in HB-EGF and enhancement of EGFR signaling. Second, phospholipase Cγ (PLCγ) activation produces diacylglycerol (DAG) from PI4,5-P2, which consecutively activates downstream PKC, phospholipase D (PLD), and phosphatidylinositol-5-kinase (PI5K). Activation of PI5K produces PI4,5-P2 from PI4-P and thus enhances the signaling. (b) Six negative feedback loops. The activation of protein tyrosine phosphatases (SHP-1 and SHP-2) inhibits EGFR signaling; the activation of ERK1 and ERK2, or ribosomal protein S6 kinase (RSK2) inhibits the son of sevenless homolog (SOS); recruitment of Casitas B-lineage lymphoma proto-oncogene (c-Cbl) by growth factor receptor-bound protein (GRB2) mediates EGFR degradation. RAS, rat sarcoma viral oncogene homolog.

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of cancer as a whole is helpful for studies on drug resistance mechanisms and clinical therapeutic strategy development.

To overcome drug resistance in cancer therapy still remains a big challenge. Deregulation of the EGFR pathway through oncogenic mutations is well recognized as an important cause of lung cancerigenesis. Several positive and negative feedback loops are located in the EGFR signaling pathway.[71] For example, two positive feedback loops have been proposed: first, ADAMs activated by proline-rich tyrosine kinase 2/v-src sarcoma viral oncogene homolog shed pro-HB-EGF and increase the HB-EGF amount, which further enhances the EGFR signaling; second, phospholipase Cγ activation produces diacylglycerol from PI4,5-P2, which consecutively activates downstream PKC, phospholipase D, and phosphatidylinositol-5-kinase (PI5K). Activation of PI5K produces PI4,5-P2 from PI4-P and thus enhances the EGFR signaling (Fig. 3a). In addition, there exist six negative feedback loops: the activation of protein tyrosine phosphatase either SHP-1 or SHP-2 inhibits EGFR signaling; the activation of ERK1 or ERK2, or ribosomal protein S6 kinase inhibits the son of sevenless homolog; and recruitment of Casitas B-lineage lymphoma proto-oncogene by growth factor receptor-bound protein mediates EGFR degradation (Fig. 3b). Additionally, multiple membrane proteins such as IGF-1R and MET acting as either direct or indirect partners for EGFR or EBB3 contribute to the transactivation of EGFR signaling (Fig. 2). The partners of MET, including integrin, the adhesive molecule CD44, class B plexins, FAS, and other tyrosine kinase receptors such as RON, are also involved in cancer initiation, progression, and malignant conversion. All of these feedback loops and signaling cross-talk strengthen and solidify the robustness of the EGFR signal pathway, which may dampen the efficiency of EGFR-targeted therapy by a single agent and trigger drug resistance sooner or later. Additionally, cancer cells harbor far more complex genetic alteration, which may make the single agent treatment unsuccessful. PIK3CA oncogenic mutations occur simultaneously with EGFR mutations.[26] Why do cancer cells need two such oncogenes to tangle and how do these two synergize in cancerigenesis? The answers to these questions may improve current therapeutic strategies. Furthermore, research into the role of stromal cells or ECM in drug resistance is still in its infancy. However, there is no doubt that future work will uncover mechanisms related to how the interaction between cancer cells and their microenvironment works together to resist the drug treatment. It is our hope that we could one day overcome drug resistance with a comprehensive view of cancer.

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Abbreviations

ADAM	a disintegrin and metalloprotease
AKT	v-akt murine thymoma viral oncogene homolog
EGFR	epidermal growth factor receptor
ERBB	erythroblastic leukemia viral (v-erb-b) oncogene
GPCR	G-protein coupled receptor
HB-EGF	heparin-binding EGF-like growth factor
IGF-1R	insulin-like growth factor-1 receptor
MET	hepatocyte growth factor receptor
NSCLC	non-small-cell lung carcinoma
PI3K	phosphatidylinositol-3-kinase
RAF	v-raf-1 murine leukemia viral oncogene homolog
RAS	rat sarcoma viral oncogene homolog
STAT3/5	signal transducer and activator of transcription 3/5
TKI	tyrosine kinase inhibitor

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