Mechanism of EGER-related cancer drug resistance

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Drug resistance in cancer arises from a complex range of biochemical and molecular events, which ultimately result in tumor cell survival. Identifying key genes and signal pathways involved in the molecular mechanisms of drug resistance is essential for establishment of new drug targets for preventing further resistance development and spreading. Epidermal growth factor receptor (EGFR) was the first growth factor receptor proposed as a target for cancer therapy. Significant progress in studying EGFR gene expression and mutation has been made in understanding the molecular events involved in EGFR-targeted agents. Recently, some individual chromosomal features such as EGFR copy number variation were demonstrated as new aspects related to drug sensitivity. Identifying these functional regulators of drug resistance will benefit therapeutic decision-making. In this study, we describe an extensive investigation of the published literature on mutation, amplification, and expression of EGFR and its downstream signaling that directly contribute to EGFR inhibitor resistance, including the gene status of KRAS, BRAF, PIK3CA, PTEN, MEK, and AKT on response to therapy. Analysis of these gene signatures identified reveals general modes of action of multicomponent therapies and the mechanisms of specific drug combinations, highlights the potential value of molecular interaction profiles in the discovery of novel therapies, and provides more information for personalized cancer medicine. *Anti-Cancer Drugs* 22:963–970 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The epidermal growth factor receptors (EGFRs) family is a family of four structurally related receptor tyrosine kinases: EGFR (ErbB-1), HER2/c-neu (ErbB-2), Her 3 (ErbB-3), and Her 4 (ErbB-4), which are involved in over 70% of all cancers [1]. The EGFR family has been investigated as a major target for cancer treatment because its activation stimulates key processes involved in tumor growth and progression, including proliferation, angiogenesis, invasion, and metastasis [2]. Upon binding to extracellular ligands, the receptors undergo conformational changes that facilitate homodimerization or heterodimerization. Receptor dimerization leads to activation of downstream signaling pathways, such as the RAS-RAF-MEK-MAPK (mitogen-activated protein kinase), phosphatidylinositol 3-kinase (PIK3CA)-AKT, and phospholipase C γ pathways and the STAT pathway [2,3].

Agents targeted against the EGFR and HER2 have been studied extensively in the laboratory, the well-identified categories include monoclonal antibodies (moAbs) and tyrosine kinase inhibitors (TKIs). Gefitinib and erlotinib are EGFR inhibitors (EGFR-Is) approved for lung cancer and pancreatic cancer, trastuzumab is an HER2 moAb, and lapatinib is a selective EGFR and HER2 inhibitor (HER2-I) approved for breast cancer [4,5]. Several agents have undergone clinical trials, such as cetuximab (Erbitux), a humanized moAb directed against the extracellular domain of the EGFR (Tables 1 and 2). Some of these drugs can disrupt EGFR signaling by competing with adenosine triphosphate (ATP) for the binding sites at tyrosine kinase domain, thus inhibiting the phosphorylation and activation of EGFR and the downstream signaling network [6,7].

Resistance to EGFR-I and HER2-I primarily arises from resistant mutations, amplification of the main target, activating mutations of downstream signaling genes, loss of function of downstream regulatory genes [4,8], and compensatory, alternative, and redundant signaling genes frequently upregulated or amplified in resistant patients [9–13]. Efflux pumps, primarily responsible for the resistance of chemotherapy drugs [14], are not expected to significantly contribute to the resistance of the evaluated drugs because these drugs are either efflux-pump inhibitors [15–17] or moAb unaffected by efflux pumps [18]. This study will focus on the gene status of EGFR and its downstream signaling effect on drug response and resistance. The analyzed genetic data include drug sensitizing mutations and copy number variations in EGFR, activating mutations in RAS, BRAF, PIK3CA, and inactivating mutations in PTEN that directly contribute to EGFR-I and HER2-I resistance in a significant percentage of patients (> 2%) [4,8]. Analysis of these gene signatures can provide more information for EGFR family-targeted therapy and can provide clues to aid in the discovery of new drug combinations and multitargeted agents.

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Table 1 Summary of anti-EGFR therapy agents

Drug name[a]	Other names	Molecular properties	Target	Approved uses	Drug manufacturer
Cetuximab	Erbitux	MoAb (chimeric IgG1)	Blocks EGFR	Cetuximab is approved by the FDA to treat the following types of cancer: squamous cell carcinoma of the head and neck and, colorectal cancer (mCRC)	ImClone Systems, Inc., Princeton, New Jersey and New York, USA
Trastuzumab	Herceptin	MoAb (human IgG1)	Blocks HER2/neu	Approved for HER2-overexpressing breast cancer	F. Hoffmann-La Roche Ltd, Basel, Switzerland
Lapatinib	Tykerb (GW572016)	Tyrosine kinase inhibitor	Both EGFR and HER2/neu (dual-TKI action)	Approved for hormone-positive and HER2-positive advanced breast cancer; approved for HER2-overexpressing breast cancer	GlaxoSmithKline, Philadelphia, Pennsylvania, USA
Erlotinib	Tarceva (OSI-774)	Tyrosine kinase inhibitor	Inhibition of EGFR	Approved for maintenance and treatment of non-small cell lung cancer. Approved for pancreatic cancer and non-small cell lung cancer	Genetech, Inc., South San Francisco, California, USA
Gefitinib	Iressa (ZD1839)	Tyrosine kinase inhibitor	Inhibition of EGFR	Approved for non-small cell lung cancer	AstraZeneca Pharmaceuticals, Wilmington, Delaware, USA

EGFR, epidermal growth factor receptors; FDA, Food and Drug Administration; mCRC, metastatic colorectal cancer; moAb, monoclonal antibody; TKI, tyrosine kinase inhibitor.
[a]These five drugs are currently being used or in clinical phase testing for anti-EGFR therapy. All of these agents are either already being used in the clinical setting or are in phase III clinical development [54,63].

Epidermal growth factor receptor-dependent mechanisms of resistance

EGFR was the first and main growth factor receptor proposed as a target for cancer therapy. Specific alterations of the EGFR gene, including somatic mutations and gene copy number variations, have been previously reported as the genetic events underlying the response to gefitinib or erlotinib (TKIs of EGFR) and trastuzumab, which is an moAb targeting the HER2/neu receptor in non-small cell lung cancer (NSCLC) and breast cancer, respectively.

Epidermal growth factor receptor overexpression

EGFR is known to be expressed more abundantly in malignant than in normal tissue. For example, 40–80% of NSCLC have a higher expression of EGFR than normal tissue [19]. EGFR expression is also important in the development and progression of malignancy. Some studies have found positive correlations among overexpression of EGFR and poorer survival [20], whereas other studies showed no correlation between EGFR expression and survival. Currently, whether EGFR protein expression (as detected by immunohistochemistry) could be used with any confidence to predict response to TKIs monotherapy remains to be determined. The response to treatment with panitumumab, which is an moAb specific to EGFR in patients with metastatic colorectal cancer (mCRC), was similar whether EGFR protein expression was high, low or negative, as assessed by immunohistochemical methods [21]. Similarly, when TKIs have been administered in combination with chemotherapy, such as in the Tarceva Lung Cancer Investigation or Tarceva Responses in Conjunction with Paclitaxel and Carboplatin trials [22] EGFR protein expression was not a reliable predictor of response to these therapies. The poor correlation between immunohistochemically determined EGFR expression and response to TKIs of EGFR may be caused by several factors. These factors include several technical aspects such as experimental equipments, the source of the supplies, errors caused by experiment operation, methodological standardization, and the interval between sample collection and preservation.

Epidermal growth factor receptor mutation

Currently, the presence of mutant EGFR in NSCLC predicts for a good prognosis, irrespective of the treatment chosen. Many of EGFR mutations are correlated with responses to TKIs both clinically and *in vitro*. The best characterized mutations are small, inframe deletions in exon 19 and a point mutation that substitutes Leu-858 with arginine (L858R) in exon 21. Inframe deletions in exon 19 that are centered on codons 756–750 make up 45–50% of EGFR mutation, and the missense mutation that converted leucine to arginine at

Table 2 Summary of the literature-reported EGFR downstream signaling that directly contributes to EGFR and HER2 inhibitors resistance, respectively, the corresponding and resistance mechanisms and relevant literatures

	Gene status	Reported resistance	Resistance mechanism	Targeted cancer
KRAS	KRAS is the most commonly mutated gene and the frequency of mutations is approximately 35–45% of colorectal cancer. The common mutations are in codon 12 or 13	KRAS mutations have emerged as a major predictor of resistance to panitumumab or cetuximab in the clinical setting	KRAS is a downstream mediator of EGFR-induced cell signaling; and KRAS mutations confer constitutive activation of Ras/MAPK pathway, (cell proliferation). Consequently, in the presence of a KRAS mutation this pathway activation cannot be significantly inhibited by an anti-EGFR moAb	CRC, NSCLC, leukemias, pancreatic cancer [64–66]
BRAF	Approximately 45% BRAF mutation in thyroid cancer, 10% in ovarian cancer, 13% in colorectal cancer. The single substitution missense mutation V600E is common	Mutations in BRAF have been recently shown to impair responsiveness to panitumumab or cetuximab in patients with mCRC	The V600E amino acid change results in constitutive activation of the BRAF kinase and promotes cell transformation [41].	CRC, melanoma, ovarian cancer, thyroid cancer [67–69]
PIK3CA	The PIK3CA gene is mutated in approximately 20% of CRCs exon 9 (E542K, E545K) and exon 20 (H1047R)	PIK3CA mutations can independently hamper the therapeutic response to panitumumab or cetuximab in mCRC [46]	The mutations constitutively activate its kinase activity and KRAS signaling pathways and allow growth factor-independent growth	CRC, lung cancer, stomach cancer [70]
PENT	Loss of expression or reduced expression	Loss of expression of the tumor suppressor PTEN protein has been reported to confer tumor resistance in patients with glioblastoma and trastuzumab resistance in patients with breast cancer	Decreased levels of the PTEN phosphatase resulted in increased PIK3CA/AKT phosphorylation and signaling	Glioblastoma, breast cancer, CRC, lung cancer [52,53]
MET	Acquired resistance can occur through amplification of the MET protooncogene. The incidence of MET amplification in TKI resistant tumor specimens was approximately 21%	MET amplification was detected in four of 18 (22%) lung cancer specimens that had developed resistance to gefitinib or erlotinib [12]	Amplification of MET causes gefitinib resistance by driving ErbB3 (HER3)-dependent activation of PIK3CA, a pathway thought to be specific to EGFR/ErbB family receptors	NSCLC [46]
AKT	Phosphorylated AKT	Compared with patients whose tumors were negative for P-AKT, patients whose tumors were positive for P-AKT had a better response rate, disease control rate, and time to progression	AKT activating mutation mediated EGFR-independent AKT signaling and contributed to EGFR inhibitor resistance	NSCLC [58]

CRC, colorectal cancer; EGFR, epidermal growth factor receptors; MAPK, mitogen-activated protein kinase; mCRC, metastatic colorectal cancer; moAb, monoclonal antibody; NSCLC, non-small cell lung cancer; PIK3CA, phosphatidylinositol 3-kinase; TKI, tyrosine kinase inhibitor.

codon 858 (L858R) in exon 21 represents 35–45% of EGFR mutation [23,24]. These activating mutations have also been found to confer sensitivity to the TKIs such as gefitinib and erlotinib [25]. For example, the response rate to TKI treatment in mutation-positive cases is 77% compared with 10% in mutation-negative cases in NSCLC [7]. The reasons for the high response rate are that these mutations lead to a higher K_m for ATP and much reduced K_i for erlotinib or gefitinib relative to the wild-type receptor. Patients with such mutations might obtain their clinical benefit because of increased receptor inhibition, which is not achieved in patients with wild-type receptors [4,26].

Clinically, the efficacy of these TKIs is often limited because of the emergence of drug resistance conferred by a second mutation, T790M, which accounts for approximately half of all resistance to gefitinib and erlotinib. When NSCLC cell lines expressing highly sensitive EGFR variants were subjected to long-term exposure to gefitinib *in vitro*, the resulting resistance to gefitinib was accompanied by the acquisition of secondary mutations in T790M substitution. The T790M mutation is the 'gatekeeper' residue in EGFR [27]. The mutation lies within the ATP-binding and drug-binding cleft of EGFR, where it results in an altered conformation that blocks entry of the TKIs and leads to clinical resistance [27]. These gatekeeper mutations may derepress the catalytic activity of EGFR and other kinases.

Epidermal growth factor receptor amplification

Gain of EGFR gene copy number was found to be associated with response to targeted agents such as trastuzumab (anti-HER2 antibody) in breast cancer [28] and potentially also to the cetuximab (anti-EGFR moAb) in NSCLC [29]; it is also associated with sensitivity to gefitinib in EFFR-positive patients [30] in NSCLC. The study by Cappuzzo *et al.* [31] indicated that patients with advanced NSCLC with EGFR amplification display higher response rates (36 vs. 3%), longer median times to progression (9 vs. 2.5 months), and prolonged overall survival (18.7 vs. 7 months) with gefitinib therapy compared with patients with wild-type EGFR [31]. The study by Moroni *et al.* [32] showed that eight of nine patients with mCRC with objective responses after treatment of cetuximab or panitumumab who were assessable by fluorescence in-situ hybridization (FISH) had increased EGFR copy number. By contrast, one of 21 nonresponders assessable by FISH had increased EGFR copy number ($P < 0.0001$ for responders vs. nonresponders). They also proposed that the response to anti-EGFR treatment has a genetic basis and suggested that patients might be selected for treatment on the basis of EGFR copy number [32,33]. Importantly, the increased EGFR gene dosage does not seem to translate into increased expression of the corresponding proteins [32,33]. Therefore, how and why copy number of EGFR gene is correlated with improved response remain largely unclear and warrant additional studies. Although increased copy number of the EGFR gene was found to be associated with response, methodologies to assess gene copy number (FISH/chromogenic in situ hybridization) still have some difficulties with technical reproducibility, and the scoring system shows high interlaboratory variability, further complicating the clinical application of this marker [34].

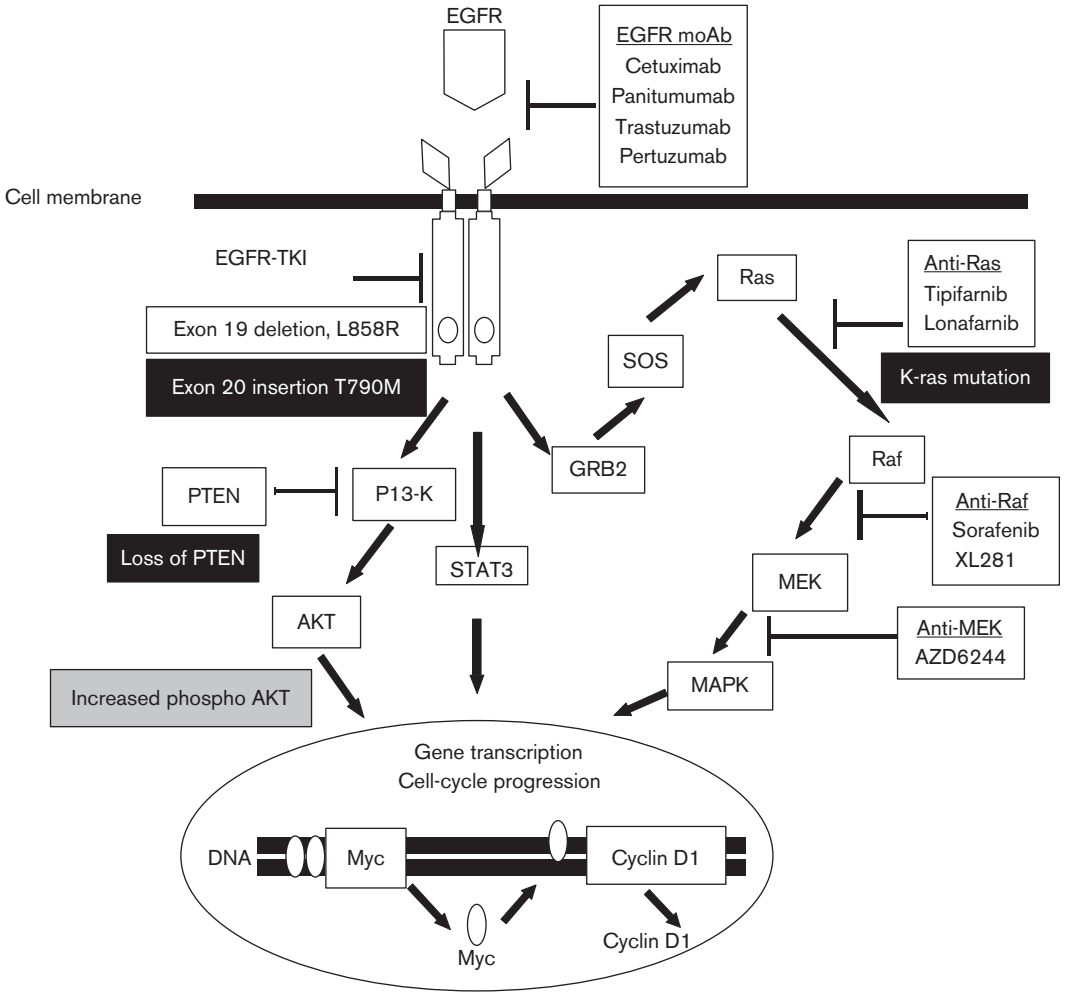
Downstream of epidermal growth factor receptor family

It has been demonstrated that the growth of many tumors is driven by constitutive activation of signaling pathways downstream of EGFR, as will be discussed below. Figure 1 shows the EGFR-related pathways involved in tumor proliferation, progression, and metastasis. On binding to extracellular ligands, an array of signaling molecules are activated, including phospholipase C, intracellular Ca^{2+}, and various protein kinase C isoforms. There are mainly two routes; on the one hand, membrane localization of the lipid kinase PIK3CA counteracts PTEN and promotes AKT1 phosphorylation, mainly involved in cell survival and motility invasion [35]. In contrast, KRAS activates BRAF, which in turn triggers MAPK. Such close interactions between these pathways may provide 'escape mechanisms' that allow tumors to circumvent a pathway that has been pharmacologically blocked. Mutation in KRAS, BRAF, or PIK3CA results in continuous activation of the downstream RAS-MAPK or PIK3CA pathways, regardless of whether the EGFR is activated or pharmacologically blocked.

KRAS is a protooncogene that encodes KRAS G-protein, which plays a critical role in the RAS/MAPK signaling pathway located downstream of many growth factor receptors including EGFR. KRAS is the most commonly mutated gene in this pathway, with mutations in 35–45% of colorectal adenocarcinomas and in approximately 20% of NSCLC [36,37]. KRAS mutations have emerged as a major predictor of resistance to panitumumab or cetuximab in the clinical setting; they are associated with a worse prognosis and a shorter survival and tend not to respond to treatment of TKI therapy. In patients with colorectal cancer, KRAS mutations are predictive for poor response to the EGFR-neutralizing antibodies, cetuximab or panitumumab. KRAS is a downstream mediator of EGFR-induced cell signaling, and KRAS mutations confer constitutive activation of the signaling pathway, independent of EGFR activation. Studies [36–38] of patients receiving first and subsequent lines of treatment have found that those with tumors carrying KRAS mutations do not respond to EGFR-targeted moAb or do not experience any survival benefit from such treatment.

The BRAF genes encode for cytoplasmic serine–threonine kinases, which are the principal effectors of

Fig. 1



Overview of interlinked cellular signaling pathways involved in the proliferation and progression of cancer. Agents targeting signaling proteins that have been evaluated or are currently being evaluated in phase II, III or IV clinical trials for cancer are shown [62]. EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; moAb, monoclonal antibody; PI3-K, phosphatidylinositol 3-kinase; TKI, tyrosine kinase inhibitor.

KRAS and are regulated by binding to BRAF [39]. Mutation in BRAF is less common compared with KRAS mutation [40]. Eighty percent of these mutations correspond to the hotspot transversion mutation T1799A that causes the amino acid substitution V600E. The other 20% accounts for a wide variable range of missense mutations and all of them reside in the glycines of the G-loop in exon 11 or in the activation segment in exon 15 near the V600. The mutation V600E confers transformant activity to the cells because it mimics the phosphorylation of T599 and/or S602 in the activation segment; hence, BRAF rests constitutively active in a KRAS-independent manner [41]. Mutations in BRAF have been recently shown to impair responsiveness to panitumumab or cetuximab in patients with mCRC [42]. Of note, KRAS and BRAF mutations are known to be mutually exclusive in colorectal cancers. The study by Di Nicolantonio *et al.* [43] retrospectively analyzed 113 mCRC tumors from cetuximab-treated or panitumumab-treated patients for KRAS and BRAF mutations. The BRAF V600E mutation was detected in 11 of 79 patients who had wild-type KRAS. None of the BRAF-mutated patients responded to treatment, whereas none of the responders carried BRAF mutations ($P = 0.029$). BRAF-mutated patients had significantly shorter progression-free survival ($P = 0.011$) and overall survival ($P < 0.0001$) than wild-type patients.

PIK3CA mutations lead to constitutive activation of p110α enzymatic activity, stimulate AKT signaling, and allow growth factor-independent growth [44]. Mutations in PIK3CA is less than or equal to 20% and mutations in PIK3CA and KRAS or BRAF may coexist within the same tumor [45]. PIK3CA mutations were significantly associated with clinical resistance to panitumumab or cetuximab. The experimental result from the study by Sartore-Bianchi *et al.* [46] indicated that PIK3CA muta-

tions can independently hamper the therapeutic response to panitumumab or cetuximab in mCRC. When the molecular status of the PIK3CA (mutation)/PTEN (loss) and KRAS (mutation) pathways are concomitantly ascertained, up to 70% of patients with mCRC are unlikely to respond to EGFR moAbs [46]. Although the study by Prenen *et al.* [47] who reported that 23 (12%) of the 200 samples carried one of the PIK3CA mutations included in their assay, is contradictory, there is no correlation between the presence of a PIK3CA mutation and impaired response to cetuximab [47]. However, the overall analysis of the published study showed that PIK3CA mutations are associated with drug resistance to a certain degree [48–50].

PTEN is a well-known tumor suppressor that counteracts the action of PIK3CA by dephosphorylating the phosphoinositide-3,4,5-trisphosphate. Loss of expression of the tumor suppressor PTEN protein, which regulates the PIK3CA-AKT signaling pathway, has also been reported to be involved in tumor resistance *in vitro* [51] and has been linked to erlotinib resistance in patients with glioblastoma [52] and to trastuzumab resistance in patients with breast cancer [53]. Many studies have demonstrated that PTEN inactivation is a negative predictor of response [54,55]. It has been recently reported that activation of the PIK3CA pathway in breast tumors with concomitant HER2 gene amplification, either through PIK3CA mutations or PTEN inactivation, underlies trastuzumab resistance. These findings indicated that assessment of PIK3CA pathway activation may provide a biomarker to identify patients unlikely to respond to trastuzumab-based therapy [56].

Other predictors

The recent studies demonstrated that resistance can occur through focal amplification of the MET protooncogene. The result from the study by Engelman *et al.* [12] showed that inhibition of MET signaling in lung cancer cells can restore their sensitivity to gefitinib. MET amplification was detected in four of 18 (22%) lung cancer specimens that had developed resistance to cetuximab [46]. Two tumors showed MET amplification in the resistant specimens but not in the pretherapy specimens, indicating that therapeutic pressure may have 'induced' this alteration or may have selected a minority of tumor cells harboring it [12]. Besides, research by Bean *et al.* [57] indicated that MET amplification occurs independently of EGFR T790M mutations and that MET may be a clinically relevant therapeutic target for some patients with acquired resistance to gefitinib or erlotinib [57].

The importance of the protein kinase B (AKT) pathway has been indicated by studies that assessed the phosphorylation status of this enzyme. The AKT is a serine/threonine protein kinase and P-AKT is the active form, which is involved in many cellular processes. The study by Cappuzzo *et al.* [58] showed that patients with P-AKT-positive tumors who received gefitinib had a better response rate, disease control rate, and time to progression than patients with P-AKT-negative tumors, suggesting that gefitinib may be most effective in patients with basal AKT activation. However, this finding has not been supported in all studies and the application of these types of molecular markers in clinical management still requires more evidence.

Conclusion

TKIs and anti-EGFR moAbs are major advances in cancer treatment. Improved understanding of these drug resistances may also allow us to stratify patients to receive treatment options that are tailored to their tumor type. It is now clear that tumor growth can be driven by constitutive activation of signaling pathways downstream of the EGFR, such as the RAS-MAPK-PIK3CA pathway. Oncogenic activation of components in these pathways can bypass the EGFR-driven signaling cascade and impair the clinical efficacy of anti-EGFR moAbs and TKIs. Such activation can occur through mutations in oncogenes such as KRAS or BRAF on one side of the EGFR-mediated pathway or by PIK3CA mutation or loss of tumor suppressor genes, such as PTEN on the opposite side of the cascade.

Collective analysis of mutation, amplification, and expression of the target genes, and drug-resistant downstream signaling and regulatory genes seem to be a reasonable approach. By development, integration, and expanding application of next generation sequencing [59], microarrays [60], and copy number variation [61] detection tools and methods coupled with other similar predictive factors and drug-resistance bypass mechanisms, a substantial number of patients could potentially be offered personalized therapeutic schemes. However, many patients respond to and/or derive clinical benefit in the absence of these candidate response predictors (i.e. gene overexpression, gene amplification, or specific somatic mutations), which indicates that additional mechanisms or indicators of response remain to be identified. These unknown factors may include transcription regulatory mechanisms and epigenetic and environmental factors. It is critically important that we continue to study other mechanisms of resistance to EGFR TKIs and moAbs so that we can provide tailored treatment to appropriately defined patients.

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Conflicts of interest

None declared.

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