Mutations and Response to Epidermal Growth Factor Receptor Inhibitors

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Abstract

Novel therapeutic agents targeting the epidermal growth factor receptor (EGFR) have improved outcomes for a subgroup of patients with colorectal, lung, head and neck, and pancreatic cancers. In these tumors, the EGFR activation turns on at least five different signaling pathways (RAS/mitogen-activated protein kinase, phospholipase C, phosphatidylinositol 3-kinase/AKT, signal transducer and activator of transcription, and SRC/FAK pathways), which are intimately interconnected, and frequent mutations involving either the receptor itself or downstream effectors have been found. Up to now, it seems that alterations at the EGFR level has major importance in EGFR tyrosine kinase inhibitor response, whereas modifications of downstream effectors could lead to treatment resistance. Furthermore, our understanding of the mechanism of the EGFR network activation provides new hypotheses on potential new anticancer drugs that may be effective.

The epidermal growth factor receptor (EGFR), a member of the HER family of receptor tyrosine kinases, has been identified as a therapeutic target for colorectal carcinoma, non-small cell lung carcinoma, head and neck squamous cell carcinoma, and pancreatic cancer. In these cancers, the Food and Drug Administration and the European Medicines Agency approved of EGFR-targeted therapies, small molecules that are tyrosine kinase inhibitors (TKI; erlotinib and gefitinib) or monoclonal antibodies (cetuximab and panitumumab) that block receptor dimerization. Overall response rate to these drugs is modest unless they are associated with chemotherapy or radiation (1). However, a subset of tumors showed drastic responses even when used in monotherapy leading to the hypothesis that tumor biology could interfere with treatment response. For TKI, it was rapidly shown in lung cancer that response was linked to the presence of mutations in the EGFR receptor tyrosine kinase domain (2, 3). In the case of monoclonal antibodies, recent translational results showed that an activation of signaling pathways downstream of the EGFR could be associated with resistance to treatment (4).

An activation of the EGFR turns on at least five differentsignaling pathways: the mitogen-activated protein kinase (MAPK), phospholipase C, phosphatidylinositol 3-kinase (PI3K)/AKT, signal transducer and activator of transcription (STAT), and SRC/FAK pathways (Fig. 1). They form an intersecting biochemical network that, when mutated, drives cell growth in a manner unrestricted by environmental cues. Phosphorylation of proteins implicated in these pathways ultimately activates transcription factors and triggers carcinogenesis through deregulation of protein synthesis, cell cycle progression, apoptosis angiogenesis, and altered metabolism (5). That is why most of the recently clinically approved or developing targeted anticancer agents focused on partners of these pathways to restrain tumor cell growth.

An activation of the EGFR network by genetic alterations (amplification or mutation) involving either the receptor itself or downstream effectors are frequent events in human cancers. The identification of genetic alterations linked to primary response and the report of secondary genetic events leading to acquire resistance to anti-EGFR therapies are still challenging. Moreover, identifying specific combinations of genetic alterations in tumors will be of importance in selecting synergistic combinations of inhibitors. As more drugs targeting transduction pathway proteins become available and as we increase our knowledge of the complexity of these signaling networks, the burden of selecting the correct drug combination for each individual cancer patient will ultimately depend on tumor alterations. This will require new diagnostic technologies and will be a major challenge over the next decade.

EGFR Alterations

For the majority of human epithelial cancers, cell growth depends on persistently activated EGFR pathway. Given this, EGFR itself was proposed as a target for cancer therapy. Autonomous EGFR activation can arise through mutational events or gene amplification. Two subtypes of EGFR mutations have been described: tyrosine kinase domain mutations and truncating mutations involving exons 2 to 7. The first group is a hallmark of non-small cell lung carcinoma and concerns 26% of tumors (average Caucasians and Asians; ref. 6). Approximately 90% of alterations affect hotspots within exons 18 to 21 that code for the tyrosine kinase domain. In frame deletions in exon 19, codons 746 to 750 account for 45% to 50% of the
cases and the substitution of leucine 858 by an arginine for at least 35% to 45%. The remaining mutants are insertions in exon 20 (5%) and rare substitutions spanning exons 18 to 21 (7). The oncogenic activities of the most commonly reported mutations have been shown (8) and they were shown to selectively activate antiapoptotic pathways through enhanced PI3K/AKT and STAT signaling (9). Finally, EGFR mutations have been more frequently found in a subpopulation of non-small cell lung carcinoma: adenocarcinoma with bronchioloalveolar features, no smoking history, women, and Asian ethnicity (10–12). The type III deletion mutation involves amino acids 6 to 273 at the extracellular domain. The mutated

Fig. 1. EGFR activation exerts its oncogenic effect via at least five pathways: the MAPK, phospholipase C, PI3K/AKT, STAT, and SRC/FAK pathways, which are intimately interconnected (green arrow). The MAPK pathway plays a key role in cell proliferation and survival. The EGFR activation leads to the recruitment of RAS, which, through RAF activation, results in a cascade of serine/threonine kinase phosphorylation (MAPK kinase and extracellular signal-regulated kinase). Phospho-extracellular signal-regulated kinase is then translocated into the nucleus where it activates transcription factors involved in cell proliferation and survival. The PI3K/AKT pathway is involved in cell proliferation. The catalytic p110 subunit of PI3K generates the second messenger PIP3 that recruits the protein kinase AKT to the plasma membrane where it is activated as a result of phosphorylation by PDK. The targets of AKT have been implicated in cell growth, angiogenesis, cell metabolism, protein synthesis, and suppression of apoptosis directly or via the activation of mTOR. The activated EGFR can interact directly with phospholipase C to activate protein kinase C, which leads to the activation of the MAPK pathway. The phosphorylation of the SRC/FAK complex can initiate the activation of the MAPK or PI3K/AKT pathway and plays an important role in cell migration. The STAT pathway activation by the EGFR is responsible for the transcription of selected genes involved in oncogenesis. This STAT activation can be induced directly by EGFR binding or indirectly through SRC-mediated EGFR signaling. Green, major interconnections between the MAPK pathway and the PI3K/AKT pathway. Ras was shown to activate PI3K, extracellular signal-regulated kinase inactivates the TSC1-TSC2 complex leading to subsequent activation of mTOR. Moreover, the EGFR pathway can be autoregulated by retrocontrol feedback loops; for example, an activation of mTOR may lead to a negative control of AKT restraining cell growth.

BC, breast cancer; CRC, colorectal cancer; EC, endometrial cancer; GB, glioblastoma; HNC, head and neck cancer; LC, lung cancer; Me, melanoma; OC, ovarian cancer; PC, pancreatic cancer; PrC, prostate cancer; TC, thyroid cancer. All the mutation data were obtained from the Sanger Institute Catalogue of Somatic Mutations in Cancer Web site (ref. 6; http://www.sanger.ac.uk/cosmic).
receptor (EGFRvIII) is ligand independent and constitutively phosphorylated. This alteration mostly concerns gliomas (38%) and was described at low level in non-small cell lung carcinoma (5%). Finally, EGFR amplification, frequently associated in lung cancer with gene mutation, has been described in many cancer types including lung, gliomas, head and neck, and colon cancers.

**MAPK Pathway Mutations**

RAS isoforms are small molecules of 21 kDa localized at the inner surface of the plasma membrane, which is encoded by the three genes KRAS, NRAS, and HRAS. They are GDP/GTP-binding proteins that act as intracellular signal transducers (13) and the GTP active form interacts with a variety of downstream effector proteins (14). RAS genes have long been known as proto-oncogenes mutated in various types of human cancers. The vast majority of these oncogenic RAS mutations affect amino acid residues G12, G13, and, more rarely, Q61 (6). They cause RAS to accumulate in the active GTP-bound state by impairing intrinsic GTPase activity and conferring resistance to GTPase-activating proteins (15).

KRAS is one of the most frequently activated oncogenes, with ~20% of all human tumors harboring an activating mutation. Activating mutations are prevalent in pancreatic ductal carcinoma (73%), colorectal adenocarcinoma (35%), lung carcinoma (17%), and endometrial and ovarian adenocarcinoma (16%). Concerning lung carcinoma, the mutation rate depends on both histological subtype (0% in small cell carcinoma, 5% in squamous cell carcinoma, and 22% in adenocarcinoma) and smoking habits (0-7% in never smokers to 30-43% in smokers; ref. 16). NRAS and HRAS are less frequently mutated (8% and 3.5%, respectively) and the type of cancers mutated is different from the KRAS mutated cancers (skin and thyroid cancer).

The RAS proteins activate proteins in the RAF1 family, which consists of the ARAF, BRAF, and CRAF serine/threonine kinases. Mutations have been described only in BRAF. A quasi-unique point mutation is observed leading to the substitution of a valine by a glutamic acid in codon 600 (V600E). Mutated BRAF proteins have elevated kinase activity and are able to transform NIH3T3 cells (17). The spectrum of BRAF mutated tumors is narrower than that observed for KRAS mutation. The most frequent mutated tumors are papillary thyroid carcinoma (46%), melanoma (45%), and colorectal carcinoma (13%; ref. 6). In this latter type of tumor, the prevalence of mutation is largely dependent on clinicopathologic variables and on the tumor phenotype (18). The prevalence varies from 3.3% in distal to 27% in the proximal part of the colon and from 5.4% in microsatellite-stable tumors up to 63% in sporadic microsatellite-instable tumors, which are associated to a defect in DNA mismatch repair system (18, 19). It is important to note that, apart from very rare exceptions, no tumor concurrently contained both BRAF and KRAS mutations (18, 19).

**PI3K/AKT Mutations**

The PIK3CA gene codes for the catalytic subunit p110α of class IA PI3K. This gene is altered in cancers by different mechanisms (mutation or amplification). Breast and endometrial cancers are probably the most frequently mutated (26-20%) followed by colon and head and neck cancers (12-15%). Mutated p110α proteins show in vitro a gain of enzymatic function by activating the AKT signaling in the absence of growth factors and are oncogenic in cell culture (20, 21). The most frequent mutant proteins (E542K, E545K, and H1047R) observed in human tumors are oncogenic in animal models (22). In colorectal cancer, PIK3CA mutations display gender and tissues specificity patterns; they occur more frequently in women and in the proximal part of the colon (19, 23), a double mutation of PIK3CA gene is observed in 6% to 9% of mutated cases (19, 24), and there is a significant concomitant occurrence of KRAS and PIK3CA mutations up to 20% (19, 25, 26).

The tumor suppressor gene PTEN encodes a dual-specificity phosphatase that has activity against lipid and protein substrates. The loss of lipid phosphatase is sufficient to cause cancer phenotype. The PTEN gene is inactivated in cancers cells by a combination of different molecular mechanisms (inactivating mutations, allelic losses, and hypermethylation of the enhancer region). It is frequently mutated in endometrial cancer (45%), gliomas (20%), and prostate cancer (13%) and more rarely in colon and lung cancer (9%). However, in colon cancer, the prevalence of mutation is clearly related to the microsatellite status. The prevalence of mutations varies from 18% for microsatellite-stable tumors (27, 28) to <2% in microsatellite-stable tumors (29), and in lung cancer, it is largely limited to small cell carcinoma (30). In colon cancer, PIK3CA gene mutation and the loss of PTEN expression in tumor cells are mutually exclusive as expected if two genes are acting in the same pathway (31).

There are three members of the AKT family (AKT1, AKT2, and AKT3), which are broadly expressed and activated by serine/threonine kinases PKD1 and PKD2. Recently, a transforming mutation in the pleckstrin homology domain of AKT1 in cancer cells has been described (32). This E17K mutation activates AKT1 by the means of pathologic localization to the plasma membrane, stimulates downstream signaling, transforms cells, and induces leukemia in mice. The prevalence of this mutation is very low besides for breast cancer. The prevalence is ~2% in colorectal cancer (32-34) and 1% in lung cancer, with a higher prevalence in the subgroup of squamous cell carcinoma (33-35). In breast cancer, the prevalence is up to 7% in ductal breast carcinoma (33). It is interesting to note that, in this cancer, AKT1 and PI3KCA mutations are mutually exclusive events as predicted if two genes act in the same pathway (31, 33, 36). In the subgroup of lobular breast cancer, the prevalence of mutation of one or the other is up to 48% (33). Amplification and mutation of AKT2 have been rarely described in colon cancer (25).

**STAT Pathway**

STAT pathway mediates signaling by cytokines and various tyrosine kinase receptors including EGFR. Constitutive activation of STAT is found in many cancers. No STAT mutations have been found, but the number of tumors screened is still low (~ 300 in cosmic database); engineered mutants were shown to activate the PI3K/AKT and MAPK pathways (37). In non-small cell lung carcinoma with constitutively activating EGFR

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3 Unpublished results.
mutation, cells survive through AKT and STAT antiapoptotic pathway activation (9). Therefore, if mutations are found in human cancers, one might expect that they may interfere with EGFR treatments.

**SRC/FAK Signaling Pathways**

The SRC gene is homologous in sequence to the avian sarcoma virus. It is the first proto-oncogene identified and its activation is frequently observed in colon and breast cancer. This activation is not due to the presence of mutation or amplification, which if they exist are probably very rare events, but to the activation of the pathway, which it belongs. Its activation induced the activation of its downstream partner FAK, which participates to cell adhesion and to epithelial to mesenchymal transition.

**Clinical-Transitional Advances**

**EGFR mutation and amplification.** The existence of EGFR genetic alterations may render tumors susceptible to inhibitors of this receptor. In lung cancer, many studies have shown that activating EGFR tyrosine kinase domain mutations are associated with drastic clinical efficacy of EGFR TKI (erlotinib and gefitinib; refs. 2, 3). Phase II studies have shown that deletion at exon 19 and L858R mutation are major markers of response to treatment in advanced lung cancer with >80% of response, and progression-free survival ranges from 7.7 to 14 months (11, 38–40). Studies showed that first-line treatment with TKI in a genotype-directed fashion to patients with advanced lung cancer with EGFR mutations results in favorable clinical outcome; again, in this group of patients, EGFR exon 19 deletions or L858R mutations were the best predictors for longer time to treatment failure (41, 42). The insertion involving exon 20, found in 5% of mutants, has not been linked to good tumor response and could be related to primary resistance (43). Patients with initial response to EGFR TKI relapse within an average of 1 year and acquired resistance is emerging as the main obstacle in managing this group of patients. Among secondary molecular events that were responsible for tumor relapse, new EGFR mutations have been characterized and the T790M has been identified in ~50% of progressing patients (44). The L747S or D761Y confer much less resistance compared with T790M (44, 45). Finally, amplification of the MET receptor and activation of IGF signaling that both activate the PI3K/AKT pathway independently of EGFR have also been shown to drive secondary resistance (46, 47). Predicting the efficacy of other EGFR TKIs (e.g., covalent inhibitors, pan tyrosine kinase inhibitors, or associations of inhibitors) is a challenge for the next years. Achieving this goal relies on the characterization of secondary events and it could be reasonable to perform rebiopsy of tumor tissue on acquisition of resistance to optimize the switch to the next line of therapy. On the contrary, the efficacy of anti-EGFR antibodies in lung cancer cell lines seems to be independent of the presence of a mutation in the EGFR tyrosine kinase domain (48), suggesting a wider efficacy of these molecules in lung cancer (49).

In other cancers, because the prevalence of EGFR mutation is very low or even absent, studies have tried to find a link between EGFR amplification and response to TKI. In gliomas, conflicting results have been published concerning the effect of EGFR gene amplification and EGFRvIII expression on response to TKI (50). In colorectal cancer, a first study described an association between an increased EGFR copy number, analyzed by fluorescence in situ hybridization, and tumor response to cetuximab (51). This association was reported with both cetuximab and panitumumab in subsequent studies (4, 52–54). However, tumor response was observed in colorectal tumors without an increase of EGFR copy number (55) and discrepant results were observed when EGFR copy number was assessed by quantitative PCR and not by fluorescence in situ hybridization (56–58). The lack of sensitivity of the PCR technique for the detection of an increase of EGFR copy number, partly due to tumor DNA dilution and the lack of reproducibility of fluorescence in situ hybridization data because of the absence of standardized EGFR scoring and heterogeneity of fluorescence in situ hybridization pattern, may explain these differences and render this molecular marker difficult to include in clinical practice (59, 60).

**MAPK Pathways**

The association between KRAS mutations and lack of response to anti-EGFR antibodies (cetuximab or panitumumab) was first shown in a small series of metastatic colorectal cancer progressing under an irinotecan-based chemotherapy (4). This result was later reported in several retrospective studies where KRAS mutations were also associated with shorter progression-free and overall survival (53, 56, 61–65). More recently, the predictive and prognostic value of KRAS mutations was confirmed in a phase III randomized controlled study comparing panitumumab to best supportive care. Patients with a KRAS mutated tumor did not respond to panitumumab and had a poorer progression-free survival than patients with a nonmutated tumor. Furthermore, panitumumab did not improve survival compared to best supportive care for patients with a KRAS mutated tumor (66). The clinical relevance of KRAS status was proven to be important not only in chemotherapy-refractory patients but also in first-line treatment. Indeed, it was shown in two randomized studies (the CRystal and Opus studies) that patients with KRAS mutated tumors do not benefit from the addition of cetuximab to a 5-fluorouracil/irinotecan-based or 5-fluorouracil/oxaliplatin-based chemotherapy in terms of response and progression-free survival (67, 68). These data led the European Medicines Agency to restrict, to patients with KRAS nonmutated tumors, the indication of anti-EGFR antibodies.

Furthermore, in lung cancer, it has also been reported that a lack of sensitivity to EGFR TKI could be linked to KRAS mutations (69–72). Moreover, it is important to note that, in both lung and colorectal cancers, not only the anti-EGFR therapies are inefficient but also an unexplained deleterious effect of the association of chemotherapy with anti-EGFR therapies with either erlotinib or cetuximab in patients with KRAS mutated tumors has been observed. Analysis of the Tribute study identified that patients with lung KRAS mutated advanced tumors have a shorter time to progression when treated with erlotinib and carboplatin plus paclitaxel compared with those treated with carboplatin plus paclitaxel alone (hazard ratio, 1.9; \( P = 0.03 \); refs. 73). Similarly in Opus study, patients with colon KRAS mutated advanced tumors have...
shorter progression-free survival when treated with cetuximab and 5-fluorouracil plus oxaliplatin compared with those treated with 5-fluorouracil plus oxaliplatin alone (hazard ratio, 1.83; P < 0.02; ref. 67).

The BRAF mutant protein seems also to confer a resistance to anti-EGFR therapies (cetuximab or panitumumab), because none of 10 published mutated patients showed response to these drugs (61, 74).

**PI3K/AKT Pathway**

The relation between the alteration of this pathway and the response to targeted EGFR inhibitors has been poorly explored. In particular, any large mutational study of the genes coding for partner proteins of this signaling pathway have been done until today. Concerning PIK3CA mutations, small series have investigated their clinical effect on resistance to EGFR inhibitors in colon cancer (4, 39, 75) and discrepant results have been observed. As PTEN negatively regulates the PI3K/AKT pathway, it is easy to speculate that PTEN inactivation downstream of the EGFR could lead to a resistance to the EGFR inhibitors. Frattini et al. showed in a series of 27 colorectal cancer patients that PTEN expression by immunohistochemistry allows to distinguish responders from nonresponder patients to cetuximab (53). With a cutoff of 50% of stained cells for positivity, all the responder patients were PTEN positive, whereas only 35% of the nonresponders had a PTEN-positive tumor. Another *in vitro* study examined the effect of cetuximab on several colon cancer cell lines and found that cell lines with loss of PTEN expression and/or PIK3CA mutation were resistant to cetuximab (76). This latter study underlines the implication of the PI3K/AKT pathway in the modulation of response to cetuximab as it was also recently suggested in a small series of colorectal cancers in which the activation of this pathway by means of PI3KCA mutation and/or PTEN allelic loss was observed in 28% of the cases, all being nonresponders to cetuximab (75). It is probable that a global activation of the PI3K/AKT pathway is more relevant than PTEN inactivation to predict response to anti-EGFR antibodies because discrepant results have been reported on the predictive value of PTEN loss of expression (77, 78), partly due to the use of immunohistochemistry, which is not yet standardized. Furthermore, we have suggested that the measurement of the phosphorylation of p70S6 kinase, which is a terminal effector of this pathway, could be a good predictor of response to cetuximab in colorectal cancer (79).

**Perspectives**

Up to now, it seems that alterations at the EGFR level have major importance in EGFR TKI response, whereas modifications of downstream effectors, especially by genetic alterations, could lead to treatment resistance.

In lung cancer, three groups of patients are emerging: one counts the patients with EGFR mutated tumors for which the use of EGFR TKI was proven to improve outcome, the second counts the patients with KRAS mutated tumors for which anti-EGFR therapies are probably not the good alternatives, and the third group counts the non-EGFR and non-KRAS mutated tumors for which response cannot be predicted. For the first group, today’s challenge is the management of acquired resistance; in some cases, higher EGFR TKI dosage was shown more active and covalent inhibitors can be used in patients with the T790M mutation. For patients with MET amplification, PI3K inhibitors could be tested or combined inhibitors could be an alternative. For KRAS mutated tumors or nonmutated tumors, treatment options will probably depend on associated alterations as PI3K mutations or STK11-inactivating mutations (a tumor suppressor gene inhibiting mTOR, mutated in 10-20% of heavy smokers). In these groups of patients, preclinical *in vitro* studies could be helpful in optimizing potential combinations and mTOR inhibitors could be of interest. Finally, finding genetic markers linked to drug response in the nonmutated tumor group is still to be investigated.

In colorectal cancer, as KRAS mutations are clearly associated with resistance to anti-EGFR antibodies, one of the major challenges is to identify, in nonmutated KRAS patients, other markers that can predict lack of response to this therapy. Among them, amplification or activating mutations of oncogenes and inactivating mutations of tumor suppressor genes described above are the most relevant candidates, such as the level of activation of EGFR downstream signaling pathway evaluated by the measurement of EGFR downstream phosphoprotein expression. Another challenge will be to find effective therapies in KRAS mutated colorectal cancer patients, which may inhibit the activation of MAPK pathway downstream of the RAS protein, as it was shown *in vitro* with Raf inhibitors but surprisingly not with MAPK kinase inhibitors (80).

**Disclosure of Potential Conflicts of Interest**

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