Cetuximab and biomarkers in non-small-cell lung carcinoma

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Abstract: Cancer progression is a highly complex process that is driven by a constellation of deregulated signaling pathways and key molecular events. In non-small-cell lung cancer (NSCLC), as in several other cancer types, the epidermal growth factor receptor (EGFR) and its downstream signaling components represent a key axis that has been found not only to trigger cancer progression but also to support advanced disease leading to metastasis. Two major therapeutic approaches comprising monoclonal antibodies and small molecule tyrosine kinase inhibitors have so far been used to target this pathway, with a combination of positive, negative, and inconsequential results, as judged by patient survival indices. Since these drugs are expensive and not all patients derive benefits from taking them, it has become both pertinent and paramount to identify biomarkers that can predict not only beneficial response but also resistance. This review focuses on the chimeric monoclonal antibody, cetuximab, its application in the treatment of NSCLC, and the biomarkers that may guide its use in the clinical setting. A special emphasis is placed on the EGFR, including its structural and mechanistic attributes.

Keywords: NSCLC, cetuximab, biomarker, cancer progression

Statistical data made available by the World Health Organization show that lung cancers are the leading cause of cancer-related mortality in both males and females worldwide.1,2 Non-small-cell lung cancer (NSCLC) is one of the two major subtypes and accounts for over 85% of cases.3 Lung cancer has been epidemiologically associated largely with cigarette smoking,4 but lifestyle, diet, passive smoking, and occupational exposure have all been found to play contributory roles.5–8 Although early stage cancer is curable, over 70% of patients present with locally advanced or metastatic disease at the time of diagnosis.9 Chemotherapy has been shown to be beneficial in patients with resected early disease and remains the backbone of therapy for patients with advanced disease, but it is notorious for its side effects,10 and the overall 5-year survival rate is still under 15%.11 Recently, the identification of drugs able to hit molecular targets has been a refreshing addition to the arsenal of tools that can be used against lung cancer.12 One such target is the epidermal growth factor receptor (EGFR), which is overexpressed in up to 80% of patients with NSCLC.13 EGFR expression in resected patient tissues has been correlated with advanced disease and poor survival.14–16 Because EGFR is overexpressed in lung cancer, it thus becomes a logical drug target; therefore, two classes of inhibitors, including monoclonal antibodies and small molecular inhibitors against EGFR, have been developed.17,18
Epidermal growth factor receptor

The EGFR is a cell surface glycoprotein receptor belonging to the ErbB family of proteins, a subfamily of four closely related proteins comprising EGFR (ErbB-1), HER2/c-neu (ErbB-2), Her 3 (ErbB-3), and Her 4 (ErbB-4).19 The extracellular domain of EGFR has four distinct subdomains consisting of two sets of tandem repeats that compose the ligand binding sites.20,21 In the absence of any ligand, the extracellular domain appears to be locked into an auto inhibitory configuration.20 Scatchard analysis indicated that the receptor possesses two different binding affinities; however, the mechanisms regulating the different binding affinities still remain obscure.22

EGFR has a single transmembrane domain consisting of 23 amino acids that attach the receptor to the membrane and function in receptor dimerization.23 Furthermore, this domain also promotes dimer formation when attached to the extracellular portion,24 and mutations in the HER2/c-neu transmembrane domain have been demonstrated to favor its dimerization.25 Moreover, exposure to a peptide corresponding to the transmembrane domain of EGFR results in the inhibition of receptor autophosphorylation and downstream signaling events.26 It is highly likely that the transmembrane domain plays a part in the alignment of the intracellular kinase domain upon dimerization.27,28

The intracellular domain consists primarily of the tyrosine kinase domain (approximately 260 amino acids), a juxtamembrane region (approximately 40 amino acids), and a carboxyl terminal regulatory region domain (approximately 232 amino acids).29 Numerous autophosphorylation sites are located on the intracellular domain of the receptor, which acts as an anchor for SH2 (Src homology 2) domain proteins.30 Analysis of its crystal structure indicated that the tyrosine kinase remains in a constitutively active configuration but is not accessible because of the regulatory region at the carboxy terminus.31,32 Ligand binding enhances the proximal alignment of the receptor, leading to trans-phosphorylation tyrosine residues favoring conformational changes that remove inhibitory constrains on the kinase domain.33 With this configuration, EGFR is now able to phosphorylate tyrosine residues on other molecules, including phospholipase C γ,34 cytoskeletal-associated proteins35 and protein 4.1.36 Moreover, residues including serine, threonine, and even tyrosine can be phosphorylated by nonreceptor kinases.37,38 These sites are phosphorylated by downstream kinases that are part of the EGFR activation cascade,30 which includes protein kinase C,39 Src,40 and PKA.41 Furthermore, phototyrosines can act as anchor sites for various proteins that contain SH-2 or phototyrosine binding domains, and they thus act as a recruitment hub for members of various cell signaling cascades.30 It is important to stress that EGFR can also copartner with other receptors outside its family and mediate signal transduction. Several studies indicated that the EGFR transactivation mechanism is subject to different regulatory influences.42,43

EGFR signaling

The binding of EGF to its receptor initiates a mitogenic signaling cascade via several pathways, including the Ras/Raf mitogen-activated protein kinase (MAPK), phosphatidylinositol 3 kinase (PI3K)-Akt, JNK, and the Stat3/Stat5 pathways as shown in Figure 1.19,44,45 These pathways converge to enhance DNA synthesis, cell proliferation, angiogenesis, invasion, and metastasis.19,44,45 Activation of the EGFR pathway orchestrates increased autophosphorylation of tyrosine kinases, which leads to a series of intracellular events culminating in enhanced metastatic propensities and angiogenesis.36

The RAS proto-oncogene family (K-ras, H-ras, N-ras and R-ras) encode four highly homologous 21 kDa membrane bound proteins involved in signal transduction. These proteins exist in either an active state bound to guanosine triphosphate (GTP) or an inactive state bound to guanosine diphosphate (GDP).47 Activating point mutations confer oncogenic potential through a loss of an intrinsic GTPase activity, resulting in an inability to cleave GTP to GDP and culminating in unabated cell proliferation downstream of EGF signaling.48 Activating RAS mutations occur in approximately 15% to 20% of NSCLCs, and in lung cancer, 90% of these mutations are located in the K-ras gene (80% in codon 12, and the remainder in codons 13 and 61), with H-ras and N-ras mutations only occasionally documented.49 K-ras mutations are mutually exclusive in EGFR and Erbb2 mutations and confer resistance to EGFR, tyrosine kinase inhibitors (TKIs), and chemotherapy.50,51 Moreover, whereas K-ras mutations are primarily observed in lung adenocarcinomas of smokers, EGFR mutations are primarily observed in lung adenocarcinomas of neversmokers.52,53

The PI3K/Akt pathway is a key regulator of cell growth, proliferation, and survival. It is commonly activated in lung cancer by changes in several of its components including PI3K, PTEN, Akt, EGFR, and K-ras.54 In lung cancer, activation of PI3K/Akt is considered a relatively early event and results in cell survival through an inhibition of apoptosis. This activation occurs either through the binding
of the SH-2 domain of P-85 to phosphotyrosine residues of activated receptor tyrosine kinase or more commonly through the amplification of PIK3CA, which encodes the catalytic subunits of PI3K.\textsuperscript{54,55} Akt, a serine/threonine kinase acting downstream of PI3K, can also have mutations that lead to pathway inactivation. In addition, PTEN regulates the PI3K/Akt pathway via phosphatase activity on phosphatidylinositol 3,4,5 triphosphate (PIP3), which is commonly suppressed in lung cancer by inactivating mutations or loss of expression.\textsuperscript{56,57}

Signal transducers and activators of transcription (STAT) are cytoplasmic transcription factors that, upon activation, translocate into the nucleus where they mediate gene expression of several downstream targets. STAT signaling is a key intrinsic pathway for cancer inflammation and is activated in tumor cells, often resulting in the induction of inflammation-associated genes.\textsuperscript{58} To date, seven STAT proteins (1–6) have been identified in mammalian cells.\textsuperscript{59} STAT proteins are important in establishing immune responses in the tumor microenvironment to promote or inhibit cancer progression. In addition, sustained activation of STAT3, and to some extent STAT5, leads to increased tumor cell proliferation, survival, and invasion.\textsuperscript{58,60} STAT3 is activated by growth factor receptor tyrosine kinases, such as EGFR and platelet-derived growth factor receptors, as well as nonreceptor tyrosine kinases, such as Src.\textsuperscript{61,62} STAT3 and STAT5 proteins have been found overexpressed in resected NSCLC tissues.\textsuperscript{63,64}

**Therapeutic options targeting EGFR in NSCLC**

Monoclonal antibodies targeting the extracellular domain of EGFR together with small molecule TKIs have been exploited pharmacologically to block EGFR activation.

Cetuximab (marketed as Erbitux\textsuperscript{\textregistered}; Dako, Copenhagen, Denmark) is a 152 kDa chimeric monoclonal antibody of the immunoglobulin G1 subclass produced in mammalian cell culture by mouse myeloma cells (Sp2/0). It was obtained by attaching the variable regions of the murine monoclonal antibody M225 against EGFR to constant regions of the human IgG1.\textsuperscript{65,66} It has two identical heavy chains consisting of 449 amino acids each and two light chains of 214 amino acids each.\textsuperscript{67} Cetuximab has a 5- to 10-fold higher affinity for EGFR than the native ligand, resulting in inhibition of the receptor function.\textsuperscript{68} It is also able to mediate antibody-dependent, cell-mediated cytotoxicity,\textsuperscript{69} and receptor interactions with other proteins such as caveolin, E-cadherin, C-Cbl, Paxillin, Crk, C-Abl, etc.
Biologics: Targets and Therapy 2012:6

Biomarkers in cetuximab therapy

The National Institute of Health (NIH) defines a biomarker as a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. In the context of cetuximab therapy, it is applicable mostly to molecules that have been or can be used to assess both sensitivity and resistance to therapy, with the goal of predicting patient subgroups that would most likely benefit or not benefit from therapy. To date, the following biomarkers have been evaluated in the assessment of the efficacy of cetuximab treatment in NSCLC.

EGFR expression, copy number evaluation, and mutation status

One of the first studies assessing combination chemotherapy with cetuximab in a Phase II trial, in which patients evenly distributed across two arms received cisplatin or carboplatin and gemcitabine with cetuximab in one arm and without cetuximab in the other arm, showed an increased response rate, progression free survival and overall survival in the cetuximab group. A similar Phase II study in which cisplatin and vinorelbine were administered with or without cetuximab also showed enhanced survival indices in the cetuximab arm. Interestingly, both studies were conducted without any preselection for EGFR status. However, a large Phase III trial investigating paclitaxel or docetaxel and carboplatin, with or without cetuximab in 676 patients with NSCLC, found no notable differences in the primary end point of progression free survival, nor in the secondary end point of overall response rate (ORR), as judged by an independent radiologic review committee when the two arms were compared. This group evaluated EGFR protein expression by IHC, gene copy number by fluorescent in situ hybridization (FISH), and EGFR mutation status by sequencing and did not find any significant difference for all of the biomarkers assessed.

The efficacy of cetuximab was also evaluated as a mono-therapy in a group of patients that were largely EGFR positive (n = 60/66), who had been previously treated with other chemotherapeutic regimens (number of prior regimens: 1 = 28, 2 = 27, and ≥3 = 11), where the response rate was found to be similar to pemetrexed, docetaxel, and erlotinib in similar groups of patients, even though these patients were heavily pretreated.

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Figure 2 The mechanism of cetuximab action.

Notes: Schematic representation of how cetuximab mediates its antitumor activity. The antibody binding to epidermal growth factor receptor prevents receptor dimerization, leading to inhibition of receptor function as shown. Cetuximab binding also fosters receptor internalization and promotes antibody-dependent cell cytotoxicity. The resulting outcomes include disruption of apoptosis, angiogenesis, proliferation, invasion, and metastasis. Abbreviation: EGFR, epidermal growth factor receptor.
The FLEX study assessed EGFR expression in the tumor tissues using immunohistochemistry and defined EGFR positivity as a prerequisite for study inclusion, with tumor immunohistochemical data available for over 99.6% of enrolled patients. An increased benefit from chemotherapy plus cetuximab was observed in patients with IHC scores greater than 150. Overall and median survival rates were also higher in the chemotherapy plus cetuximab group as compared to the chemotherapy alone group in patients with high EGFR expression.\(^8\)

In a somewhat different approach, a Phase II clinical trial evaluated EGFR gene copy number by FISH as a predictive index for cetuximab efficacy. This trial enrolled 229 patients with advanced NSCLC, who were divided into two groups receiving paclitaxel and carboplatin with or without cetuximab, and found a significantly higher survival benefit in FISH-positive EGFR patients. Median progression-free survival and median survival time were both twice as long in the FISH-positive patients as in the FISH-negative patients, whereas the disease control rate was 81% in FISH-positive and 55% in FISH-negative patients, respectively. Complete response/partial response was also numerically higher in FISH-positive (45%) versus FISH-negative (26%) patients.\(^9\)

A meta-analysis looking at four trials in which 2018 previously untreated NSCLC patients were ultimately analyzed concluded that cetuximab improved overall survival and overall response rate.\(^10\) The meta-analysis did not consider any biomarkers, but nonetheless tried to show the overall benefits of cetuximab. Nevertheless, however, our critical review of all studies on EGFR showed that EGFR currently cannot be considered a reliable biomarker for consistent response in NSCLC.

\textbf{K-ras}
In the abrogation of the EGFR function, it became evident that in addition to EGFR, other key downstream molecules were equally important. Several reports have shown that constitutive activation of key downstream components renders the EGFR blockade by antibodies and/or TKI ineffective. One of these essential downstream factors is the small G protein proto-oncogene K-ras (Kirsten rat sarcoma 2 viral oncogene homologue). K-ras can acquire activating mutations in exon 2, thus isolating the pathway from the effect of EGFR and rendering EGFR inhibitors ineffective.\(^11–14\) K-ras mutations are mutually exclusive to EGFR mutations in NSCLC and are more associated with smoking.\(^15,16\) Generally, K-ras mutations have been tied to poor outcomes, as confirmed by a meta-analysis of 28 studies encompassing all stages of NSCLC, which showed that tumors with K-ras mutations were associated with poor prognosis.\(^47\)

In the bid to appraise the use of K-ras as a predictive biomarker in cetuximab therapy, however, a few clinical trials have integrated its analysis into their protocols. The Khambata-Ford et al trial discovered that 17% of the patients from their patient pool harbored K-ras mutations, as detected by sequencing. However, they were unable to find any significant clinical correlation that could be attributable to this biomarker in the context of cetuximab treatment.\(^76\) Reevaluation of tissues used for the FLEX study for KRAS mutations in codons 12 and 13 with polymerase chain reaction-based assays revealed 17% positive samples out of 375 patients, in which however, they found no significant predictive or prognostic outcomes differentiating mutant and nonmutant K-ras cases with the administration of cetuximab.\(^87\) A Phase II selection design trial of chemotherapy together with concurrent or subsequent cetuximab addition in advanced stage NSCLC patients evaluated the significance of K-ras and did not find any significant association with any efficacy parameters in both K-ras and wild-type patients.\(^88\)

\textbf{Phosphatase and tensin homolog (PTEN)}
Thus far, only one study has sought to evaluate PTEN as a biomarker for cetuximab therapy in NSCLC. Data stemming from the FLEX study discussed above found 196 out of 303 patients to be PTEN-positive as assessed by immunohistochemical analysis. Patients in both the cetuximab-plus-chemotherapy and chemotherapy-alone groups experienced better overall survival if their tumors expressed PTEN compared with those whose tumors were negative for expression. Although this finding was not significant, the authors still inferred that the absence of PTEN expression might be a marker of poor prognosis.\(^87,89\)

\textbf{Biomarkers in ongoing clinical trials}
At present, a number of clinical trials are still evaluating the efficacy of cetuximab in combination with other treatment modalities inclusive of radiotherapy, in combination with TKI, and other chemotherapeutic drugs (Table 1). Most of these trials are also assessing biomarker status that could be predictive of prognostic value. These trials are studying EGFR expression, KRAS in individualized or combined trials with fluorodeox-D-glucose-position emission tomography/computed tomography, MAPK, phosphorylated AKT, p27, and Ki-67 biomarkers.
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Abbreviations: EGFR, epidermal growth factor receptor; NSCLC, non-small-cell lung carcinoma; IHC, immunohistochemistry; FISH, fluorescence-in-situ hybridization; FDG-PET, fluorodeoxyglucose positron emission tomography (FDG PET); CT, computed tomography; pMAPK, phosphorylated mitogen activated protein kinase; K-ras, Kirsten rat sarcoma viral oncogene homolog.
Putative biomarkers in preclinical studies
In a quest to find alternative biomarkers for cetuximab in NSCLC, especially in cases of cetuximab resistance, several groups have employed different strategies to identify and analyze markers of resistance. In one such study, our group showed that low E-cadherin and high urokinase-type plasminogen activator receptor (u-PAR), but not EGFR, was associated with resistance to cetuximab in seven NSCLC cell lines. This same expression pattern was also observed in 63% of patients with progressive disease while on cetuximab therapy, implying that low E-cadherin and high u-PAR are markers of cetuximab resistance. Furthermore, Yonesaka and colleagues convincingly showed that ERBB2 amplification is a unique mechanism of drug resistance to cetuximab in NSCLC. Interestingly, they were able to show that ERBB2 amplified NSCLC cells remain sensitive to gefitinib, but not to cetuximab. Therefore, a phenomenon likely to be associated with gefitinib but not cetuximab is also able to inhibit ERBB2 in clinically achievable concentrations. The expression of amphiregulin (a growth factor regulator related to EGFR and transforming growth factor-alpha) expression was also found to predict sensitivity to cetuximab in EGFR wild-type cancers.

Discussion
Conflicts of interest exist between pharmaceutical companies that want to market their drugs, physicians that are looking for drugs that will best meet the needs of their patients, and the very ill patients that want to get better at all costs. A good predictive biomarker would be an optimal solution to these conflicts of interest. However, as experience has shown, robust, valid, sensitive, and specific biomarkers are few and far between. In the case of cetuximab, overwhelming evidence, particularly in meta-analysis studies, has shown that patients with advanced NSCLC derive benefit, especially when this antibody is combined with cytotoxic chemotherapy. Cetuximab has even been found to be of benefit after the failure of gefitinib, regardless of EGFR mutational status.

Existing data on EGFR protein expression, evaluated through IHC, suggested that patients with high scores stand to gain when cetuximab is included in the therapy, which supports its further evaluation as a candidate biomarker. Tangible evidence, however, also exists showing that not all patients benefit. The problem with IHC is that protocols vary, which could have considerable consequences on the outcome. Even in the FLEX study, where this biomarker was evaluated, in an effort to obtain a high degree of consistency in the analysis, all the individuals involved in the interpretation of the results had to be collectively tutored.

The EGFR gene copy number detected by FISH may be another potential biomarker for the selection of NSCLC patients for treatment with EGFR-directed therapies. Patients with FISH-positive tumors have demonstrated a higher disease control rate compared to patients with FISH-negative tumors. Furthermore, survival favored FISH-positive patients receiving concurrent therapy. In the BMS099 trial, EGFR FISH positivity was seen in 54 of 104 (52%) patients, but was neither prognostic nor of predictive value with regard to cetuximab efficacy. Thus, EGFR FISH positivity as a predictive factor of benefit from cetuximab therapy remains undecided and needs further exploration.

It was interesting to observe that with small molecule TKIs, a preferential response was observed in females, patients with adenocarcinomas, Asians, and neversmokers. A more in-depth analysis of these subgroups revealed that specific activating mutations in the tyrosine kinase domain of the EGFR gene were responsible for the observed benefit from TKIs. In agreement with the findings in NSCLC cell line studies that these mutations were associated with sensitivity to gefitinib but not cetuximab, these mutations did not seem to affect patients’ response to cetuximab in the Phase III trials. In addition, no significant treatment-specific correlations between EGFR mutation status and progression-free survival, overall survival, or response rate were observed in the BMS099 trial. Therefore, it is safe to conclude that EGFR mutations are not useful as biomarkers in cetuximab therapy.

In colorectal cancer, KRAS mutation status was found to be a useful marker of resistance because the benefit of cetuximab was found to be limited to patients with KRAS wild-type tumors. The results from two Phase II trials that compared platinum-based chemotherapy with cetuximab (concurrent/sequential) and with bevacizumab in patients with advanced NSCLC, however, showed no differences in progression-free survival or overall survival with cetuximab in relation to K-ras mutation status. The Phase III trials that evaluated K-ras mutations in NSCLC also found that K-ras mutation status had no impact on progression-free survival, overall survival, or response rate in relation to cetuximab administration. The observed differences between colorectal and NSCLC might be the result of alternative routes of signal transduction in NSCLC that render K-ras insignificant and impressively show that every tumor entity needs to be individually considered when establishing biomarkers for novel therapeutics. An increasing number of
putative biomarkers are still in the preclinical pipeline, and it will be interesting to see which ones will be brought over into clinical trials. Thus far, the evidence of their potential use is limited to a few studies, which need to be reinforced by more groups and follow-up studies.

Conclusion
EGFR protein expression still has the potential to advance to a biomarker that is able to predict favorable outcomes from cetuximab administration in NSCLC. It is rather disappointing that with all the advances in molecular cancer research and the several clinical trials that have evaluated cetuximab in the context of NSCLC, no clear significant biomarker has so far been discovered. A major contributing reason that could account for the observed findings is that the methods for the detection of EGFR positivity as well as other potential biomarkers are by no means standardized, which leaves room for a great deal of improvement. Secondly, the analysis conducted on some of the biomarkers as in the case of K-ras mutations was done on a small subset of patients, where statistical validity is difficult to establish. However, the possibility of other unidentified molecular mediators important in the response of NSCLC to cetuximab cannot be ruled out, and only when these mediators are identified can the desired outcomes be achieved. Moreover, in our opinion, the clinical trials do not all have the same endpoints regarding the survival indices being evaluated. To resolve these issues, multicentered clinical trials should be organized such that a significant number of patients with similar clinical backgrounds are included and the approach/method used for biomarker detection is standardized. Ongoing clinical trials and preclinical studies indicate that more biomarkers could soon be in contention.

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