Epidermal Growth Factor Receptor Activation: How Exon 19 and 21 Mutations Changed Our Understanding of the Pathway

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Abstract The discovery of epidermal growth factor receptor (EGFR) mutations in never-smokers has been the most relevant finding ever in non – small cell lung cancer. When patients whose tumors bear the sensitizing mutations are treated with the tyrosine kinase inhibitors gefitinib or erlotinib, we witness response rates and durations never before reported, including complete responses. At the same time, the presence of EGFR mutations has raised numerous new questions, tantalizing data, and new challenges for treatment. This is particularly true as we try to generalize the findings in lung cancer to other malignancies. The indiscriminate use of gefitinib or erlotinib in the general lung cancer population results in meager survival benefit for patients. Similarly, the tyrosine kinase inhibitors have limited activity in a variety of tumor types with EGFR overexpression. This has led to the question of whether EGFR remains a viable target in patients other than those whose tumors contain mutations, and whether the modest activity of cetuximab in colorectal cancer and head and neck cancer represents all that we can expect from inhibition of this pathway in the absence of mutation. Mechanisms of pathway activation other than mutation have been discovered in recent years, and include overexpression mediated by gene amplification or by amplification of a dinucleotide repeat in the EGFR promoter, mutation of an extracellular region on EGFR generating a mutant protein termed EGFRvIII, and enhanced signaling due to heterodimerization with other members of the EGFR family, particularly overexpression of HER2/HER3. The extent to which these paths to EGFR activation will confer sensitivity to the tyrosine kinase inhibitors or to EGFR monoclonal antibodies is being explored. Thus far, published clinical data suggest that there is little room for the administration of gefitinib or erlotinib in the absence of EGFR mutations. The five articles in this edition of *CCR Focus* will address the various mechanisms of EGFR pathway activation and provide insight into the potential for translation into clinical relevance.

Protein tyrosine kinases are enzymes that catalyze the transfer of phosphate from ATP to tyrosine residues in polypeptides. They regulate cellular proliferation and survival. Receptor tyrosine kinases are transmembrane proteins with a ligand-binding extracellular domain and a catalytic intracellular kinase domain. The kinase domains have a bilobar structure, with an NH_2-terminal lobe that binds ATP and magnesium, a COOH-terminal lobe containing an activation loop, and a cleft between the lobes to which polypeptide substrates bind (1).

The epidermal growth factor receptor (EGFR) pathway was first identified as a potential target for anticancer therapy in the early 1980s, and in recent years therapies directed at this target have been added to the arsenal available to the clinical oncologist. Based on a large number of clinical trials, some of which are summarized in Table 1, the U.S. Food and Drug Administration has granted limited approval for the use of these agents. The U.S. Food and Drug Administration first approved gefitinib in May 2003 and later erlotinib for the therapy of lung cancer, the latter based on a 2-month improvement in survival (2, 3). Subsequently, the U.S. Food and Drug Administration approved the use of the anti-EGFR monoclonal antibody cetuximab in combination with irinotecan chemotherapy in colorectal cancer based on an improvement in survival that was <2 months (4). Cetuximab in combination with radiation therapy in head and neck cancer was added to the drug labeling in March 2006. Although the gefitinib approval was originally based, in part, on the long duration of response in patients who experienced objective responses, in June 2005 the scope of approval for gefitinib was limited based on results of a randomized trial that did not confirm a survival benefit. The overall low response rates to EGFR inhibitors in lung and colorectal cancer and the lack of activity against a variety of other solid tumors that many had predicted would be susceptible to this strategy have been disappointing but have acted as a catalyst to better understand the molecular changes underlying these results, in particular the successes.

Noteworthy successes have led to new understandings of how this pathway might be targeted. Based on the experience in

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Table 1. Selected clinical trials using EGFR targeted agents

Agent	Tumor type (study identifier)	Trial design	Response rate (%)	Clinical benefit	Reference
Erlotinib vs placebo	NSCLC (BR.21)	Phase III	8.9	PFS, 2.2 vs 1.8 mo; OS, 6.7 vs 4.7 mo	(7)
Gefitinib vs placebo	NSCLC (ISEL)	Phase III	8 vs 1	PFS, 3 vs 2.6 mo; OS, 5.6 vs 5.1 mo	(60)
Gefitinib	NSCLC (IDEAL2)	Phase II	10	MDR 7 mo; OS, 6 mo	(61)
Gefitinib	BAC (SO126)	Phase II	17	OS, 13 mo	(62)
Cetuximab	Colorectal cancer	Phase II	9	OS, 6.4 mo	(63)
Gefitinib	Colorectal cancer	Phase II	0	8 SD	(64)
Erlotinib	Head and neck cancer	Phase II	4.3	PFS, 2.2 mo	(65)
Gefitinib	Head and neck cancer	Phase II	1.4	PFS, 1.8 mo; OS, 5.5 mo	(66)
Erlotinib	Ovarian cancer	Phase II	6	OS, 8 mo	(67)
Erlotinib	Hepatocellular cancer	Phase II	7.8	OS, 13 mo	(68)
Cetuximab	Renal cell cancer	Phase II	0	TTP, 57 d	(69)
Gefitinib	Renal cell cancer	Phase II	0	PFS, 2.7 mo	(70)
Erlotinib	Breast cancer	Phase II	0	—	(71)
Gefitinib	Breast cancer	Phase II	1.7	OS, 12 mo	(72)
Gefitinib	Breast cancer	Phase II	0	OS, 16.5 mo	(73)
Gefitinib	Mesothelioma	Phase II	4	OS, 6.8 mo	(74)
Combination regimens					
Gemcitabine and cisplatin ± erlotinib	NSCLC TALENT	Phase III	31.5 vs 29.9	TTP, 5.9 vs 6.1 mo; OS, 10.7 vs 11.0 mo	(75)
Paclitaxel and carboplatin ± erlotinib	NSCLC TRIBUTE	Phase III	21.5 vs 19.3	TTP, 5.1 vs 4.9 mo; OS, 10.6 vs 10.5 mo	(46)
Gemcitabine and cisplatin ± gefitinib	NSCLC INTACT1	Phase III*	49.7, 50.3, 44.8	TTP, 5.5, 5.8, 6.0 mo; OS, 9.9, 9.9, 10.9 mo	(76)
Paclitaxel and carboplatin ± gefitinib	NSCLC INTACT2	Phase III*	30.0, 30.4, 28.7	TTP, 4.6, 5.3, 5.0 mo; OS, 8.7, 9.8, 9.9 mo	(77)
Irinotecan ± cetuximab	Colorectal cancer	Phase III	22.9 vs 10.8	TTP, 4.1 vs 1.5 mo; OS, 8.6 vs 6.9 mo	(4)
FOLFIRI + Erlotinib	Colorectal cancer	Phase I	—	Too toxic	(78)
Oxaliplatin + gefitinib	Colorectal cancer	Phase I/II	0	38% SD at 12 wk	(79)
XRT ± cetuximab	Head and neck cancer	Phase III	Locoregional control (mo) 24.4 vs 14.9	OS, 49 vs 29.3 mo, 17.1 vs 12.4 mo	(80)
Cisplatin ± cetuximab	Head and neck cancer	Phase III	26 vs 10	PFS, 4.2 vs 2.7 mo; OS, 9.2 vs 8.0 mo	(81)
Erlotinib ± temozolomide	Glioma	Phase I	PR 8/57	Six pts with PFS >6 mo	(82)
Cetuximab + gemcitabine	Pancreatic cancer		12.2	OS, 13.9 mo w/rash vs 2.3 mo w/o rash	(83)

Abbreviations: BAC, bronchioloalveolar carcinoma; MDR, median duration of response; TTP, time to progression; PFS, progression-free survival; OS, overall survival; SD, stable disease; PR, partial response; FOLFIRI, folinic acid, fluorouracil, and ironotecan.
*INTACT1 and INTACT2 randomized patients to 500 mg/d gefitinib, 250 mg/d gefitinib, or placebo.

breast cancer where ErbB-2 overexpression predicts response to trastuzumab, many had assumed that overexpression of EGFR would confer responsiveness to EGFR tyrosine kinase inhibitors. However, as reviewed by Riely et al. (5) in this issue of *CCR Focus*, this expectation has not been borne out by clinical trials (2, 3). Instead, it seems that in lung cancer, response to tyrosine kinase inhibitors is directly linked to aberrant EGFR tyrosine kinase signaling due to activating mutations that are inferred to be involved in the etiology and maintenance of the malignant phenotype. This recognition created a new paradigm that the EGFR pathway must be activated in cancer to serve as a therapeutic target; in other words, the cell must be "addicted" to the EGFR pathway (6).

As with any true discovery, the identification of activating EGFR mutations in never smokers with lung cancer brought more questions than answers. What range of EGFR activating mechanisms are directly involved with the malignant phenotype and potential targets? Will another cancer subset be found with activating EGFR mutations in the kinase domain? If tyrosine kinase mutations are exclusive to lung cancer in nonsmokers, what mechanism underlies the development of that mutation? Second-hand smoke? Infection? What mediates "uncomplicated" EGFR overexpression and can mere overexpression promote or maintain the malignant phenotype? What other mechanisms of EGFR activation can also confer sensitivity to EGFR inhibitors? How does an investigator wanting to inhibit the EGFR pathway choose which agent to study? Will different mechanisms of action of EGFR inhibitors be required to affect the diverse mechanisms of activation? Why have combinations of tyrosine kinase inhibitors with chemotherapy failed thus far whereas combinations with the monoclonal antibodies have apparently succeeded? Can tyrosine kinase inhibitors be developed that inhibit the tyrosine kinase inhibitor–resistant EGFR mutant? This issue of *CCR Focus* is aimed at reviewing

the diverse mechanisms of EGFR activation as the basis for beginning to understand some of these questions.

Clinical Studies and the Identification of EGFR Mutations in Specific Subgroups of Patients

The BR.21 study, on which the erlotinib approval was based, was a phase III trial involving patients who had had progression after standard chemotherapy for non–small cell lung cancer (NSCLC; ref. 7). Patients were randomly assigned in a 2:1 ratio to receive either 150 mg of erlotinib daily or a placebo. The response rate was 8.9% in the erlotinib group and <1% in the placebo group. Progression-free survival was 2.2 and 1.8 months, respectively. Overall survival was 6.7 and 4.7 months, in favor of erlotinib (7). In the multivariate analysis, adenocarcinoma, never having smoked, and EGFR

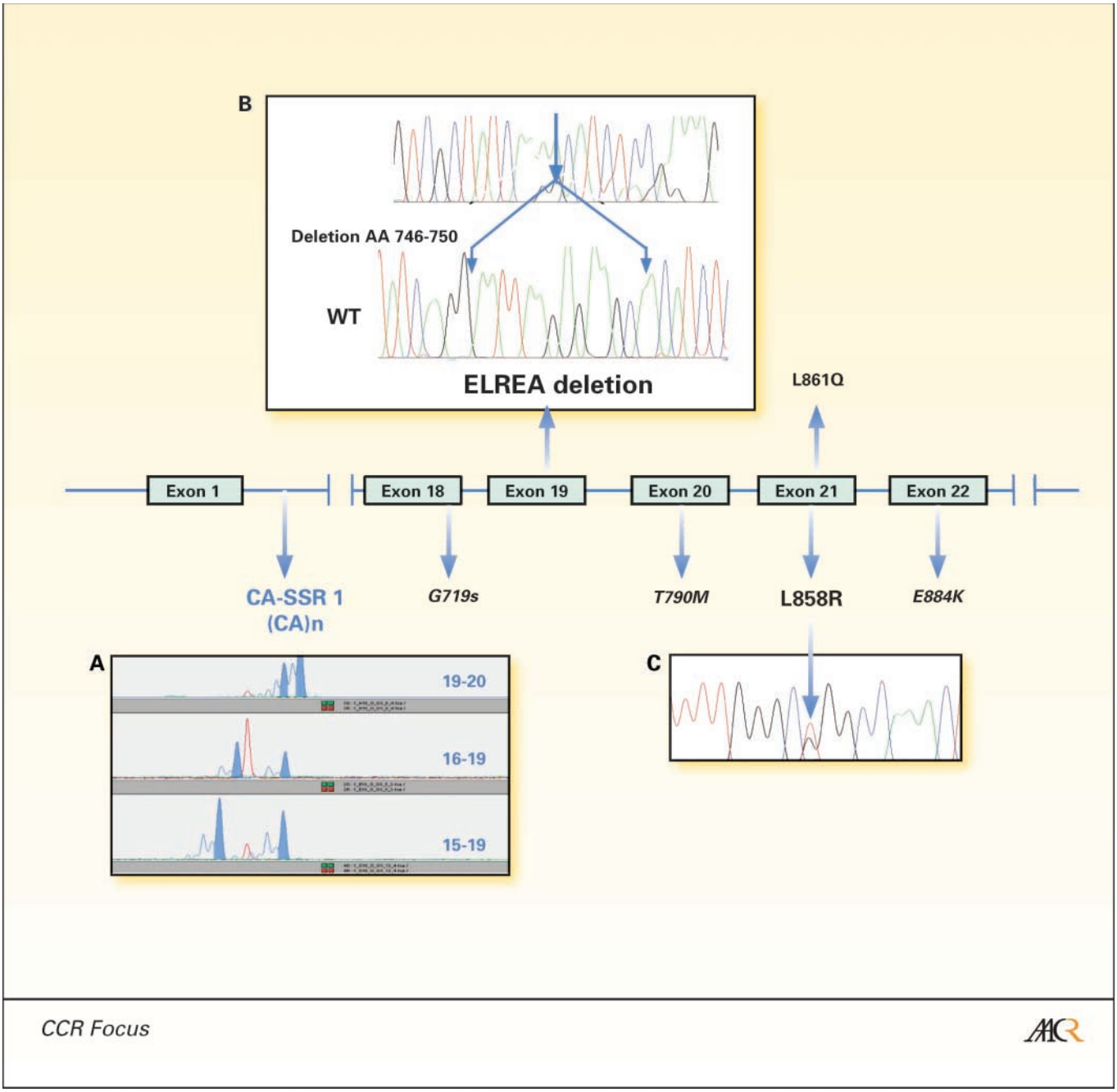


Fig. 1. Diagram showing the most common *EGFR* gene alterations and polymorphic CA dinucleotide repeat (CA-SSR 1). *A,* example of CA-SSR 1 alleles from three different patients analyzed in DNA isolated from peripheral blood lymphocytes using PCR and fluorescent-based capillary electrophoresis. *B,* electropherogram from exon 19 deletion (ELREA amino acids in position 746-750) and wild-type DNA analyzed by direct sequencing. *C,* electropherogram from a heterozygous L858R point mutation (CTG to CGG) in exon 21. Other less common mutations are also indicated.

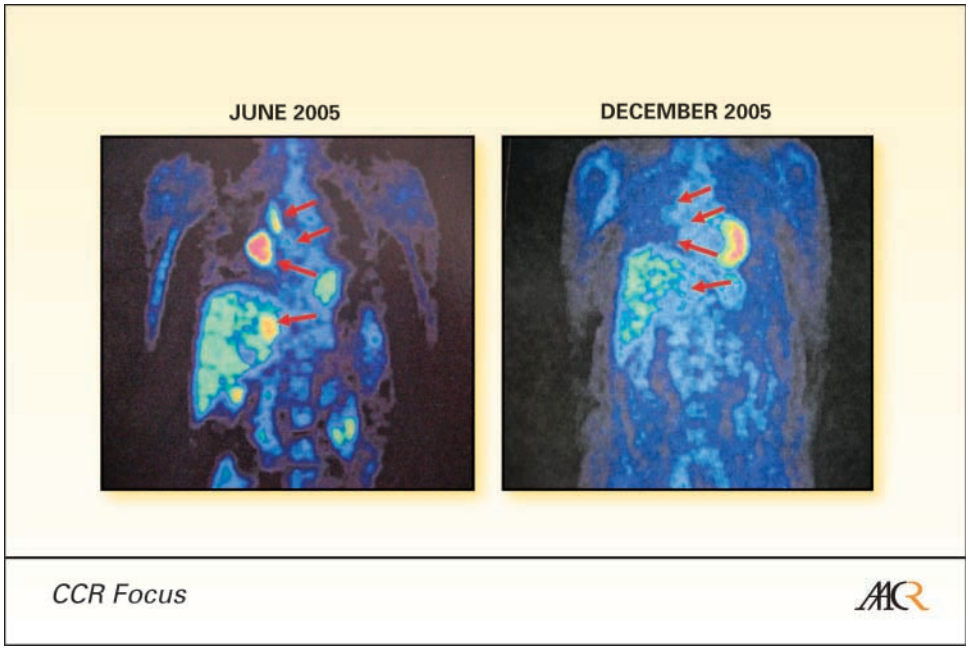


Fig. 2. Positron emission tomography scans of a 62-year-old female, never-smoker, with lung adenocarcinoma and liver metastases. The patient harbored an EGFR deletion mutation in exon 19. A durable complete remission was attained with erlotinib.

expression were associated with response (7). However, the magnitude of the benefit was meager: the response was 14% in adenocarcinomas, 25% in never-smokers, and 11% in tumors with positive EGFR expression by immunohistochemistry (7). The approval of erlotinib for all lung cancers, without the benefit of reliable predictive markers, effectively accepted a less than optimal treatment for NSCLC patients, regardless of the cost. However, the discovery of somatic mutations in the tyrosine kinase domain of the *EGFR* gene in lung adenocarcinomas responding to gefitinib or erlotinib has provided a means to resolve this problem. Combined data from four original studies indicate that 80% of NSCLCs responding to gefitinib or erlotinib harbor in-frame deletions or missense mutations in the EGFR tyrosine kinase domain, compared with none of 36 refractory tumors ($P < 0.05$; refs. 8–11). Among 617 NSCLCs screened by Shigematsu et al. (12), EGFR mutations were found in the same subgroups responding to gefitinib or erlotinib: 50% of never-smokers versus 10% of ever-smokers and in 40% of adenocarcinomas versus 3% of other histologies. As reviewed by Riely et al. (5) in this *CCR Focus*, ~85% to 90% of these mutations occur at exons 19 and 21 near the ATP cleft of the tyrosine kinase domain, where 4-anilinoquinazoline compounds, such as gefitinib, compete with ATP for binding. The most common mutations in the *EGFR* gene are an exon 19 deletion that eliminates a leucine-arginine-glutamate-alanine motif in the tyrosine kinase domain of EGFR and a thymine-to-guanine transversion that results in an arginine-for-leucine substitution at amino acid 858 (L858R) in exon 21 (Fig. 1). A wealth of data indicates that these and other EGFR mutations at exons 19 and 21 may be useful molecular markers for selecting treatment in NSCLC (8–11, 13–18). We reported the case of a Spanish female with recurrent lung adenocarcinoma, multiple brain metastases, and severe neurologic impairment, who received gefitinib through a gastric feeding tube and experienced a rapid recovery that has lasted more than 2 years. Her primary lung tumor contained a deletion mutation (13). This exemplifies the dramatic responses, never before seen, that frequently occur in patients whose tumors harbor EGFR mutations. In our series of patients enrolled in Spanish Lung Cancer Group trials, among more than 300 patients screened, EGFR mutations have been found in 38% of never-smokers, 10% of ex-smokers, and 4% of current smokers. The first 28 patients with EGFR mutations attained a 90% response rate. Several complete responses, including patients with liver metastases (Fig. 2), have been observed among lung cancers of never-smokers, whereas partial responses are equally distributed among never-smokers and ex-smokers (19).

Downstream Effects of Inhibition of EGFR Activated by Mutation

Until these activating mutations were discovered, the hope was that in solid tumors EGFR inhibitors would impair survival mechanisms, thereby leading to greater sensitivity to therapy, either of single agents targeting the EGFR pathway or of combination therapies. However, the disappointing results of combination therapies have shifted the focus to understanding how interdicting these activating mutations can bring about cell death, issues that are covered in this issue of *CCR Focus* by Ono and Kuwano (20). *In vitro* studies have provided partial insights. For example, the H3255 cell line harboring an L858R mutation was originally established from cells in a malignant pleural effusion obtained from a female nonsmoker with lung adenocarcinoma. This cell line is 50 times more sensitive to gefitinib than most other cell lines. Treatment of H3255 cells with 100 nmol/L gefitinib inhibits EGFR autophosphorylation as well as phosphorylation of known downstream targets of EGFR including the extracellular signal–regulated kinase 1/2 and AKT (10). Consistent with this, Ono et al. (21) also showed

that sensitivity in the highly gefitinib-sensitive PC9 cell line, carrying a deletion mutation (E746-A750), correlated with the phosphorylation of extracellular signal–regulated kinase 1/2 and AKT. These observations have led to the hypothesis that constitutive AKT activation is associated with the presence of EGFR mutations and, notably, that AKT activation may lead to chemoresistance to cytotoxic drugs, including taxanes and cisplatin (22, 23). Biochemical assays have shown that cells with EGFR mutations are 100-fold more sensitive to gefitinib than cells with wild-type receptors and exhibit markedly increased resistance to doxorubicin and cisplatin (23). To date, however, the clinical observations do not support this hypothesis as evidenced by the randomized trials of gefitinib and erlotinib in combination with chemotherapy that show neither benefit nor disadvantage from adding EGFR inhibitors to chemotherapy (2). Whether similar results would be obtained following combination of EGFR inhibitors with chemotherapy in the subset of patients with EGFR mutations is not known, nor whether benefit could accrue from combining cetuximab instead of the tyrosine kinase inhibitors with chemotherapy in lung cancers.

All EGFR Tyrosine Kinase Mutations Are Not Equivalent

Further work is required to better understand the significance of individual mutations, a topic covered in more detail by Riely et al. (5). Biochemical examinations have indicated that only cells harboring L858R, E746-A750 deletion, and G719S mutants (Fig. 1) were clearly more sensitive to gefitinib than wild-type EGFR–expressing cells. Intriguingly, S768I and E709G lacked ubiquitination and had hyperphosphorylation at Tyr^{1045} and were actually more resistant to gefitinib. Biochemical analysis showed that phosphorylation at Tyr^{1045} was very weak in the deletion mutants and relatively weak for L858R. These biochemical properties are in agreement with clinical findings by Mitsudomi et al. (15) that deletions predict better response to gefitinib than the L858R mutation. Among 291 NSCLC patients (24), mutations were found in 54% of never-smokers, 43% of ex-smokers, and 3% of smokers. For patients with EGFR mutations, the time to progression after gefitinib or erlotinib was 12 months and median survival was 20 months. However, patients with deletions had a time to progression of 12 months, compared with 5 months for patients with L858R ($P = 0.01$); patients with deletions had a median survival of 34 months, compared with 8 months for patients with L858R ($P = 0.01$). These findings are extremely important and require further evaluation (Fig. 3).

Selecting Patients for EGFR Mutation Assessment

The role of EGFR mutation assessment in clinical oncology is a paradigm in evolution, as addressed by Riely et al. (5) in this issue of *CCR Focus*. Whereas a consensus on specific guidelines for mutation analysis does not exist at the present time, the evidence indicates that indiscriminate testing for EGFR mutations in all NSCLCs may be unnecessary because these mutations are rarely found in squamous cell carcinomas, none in 454 cases (25). This is borne out by our experience assessing 900 Spanish NSCLC patients, in whom EGFR mutations were found only rarely in current smokers compared with 38% in never-smokers (Table 2).[3] Marchetti et al. (25) found EGFR mutations in 10% of 375 adenocarcinomas; we found mutations in 15% of adenocarcinomas; a similar frequency has been reported in a smaller series of Spanish patients (26). In contrast to findings by other authors (11, 25), we have found mutations in 15% of large-cell carcinomas. Whereas mutational analysis of EGFR exon 19 deletion mutations and EGFR L858R point mutations by PCR-based methods using paraffin blocks could become the cornerstone of laboratory testing (27), additional markers may also be queried. The issue of who should be tested is likely to remain controversial until a definitive test is established; there is substantial evidence to suggest that current detection techniques are not sufficiently sensitive to all EGFR mutations, particularly in the presence of normal tissue contamination (28).

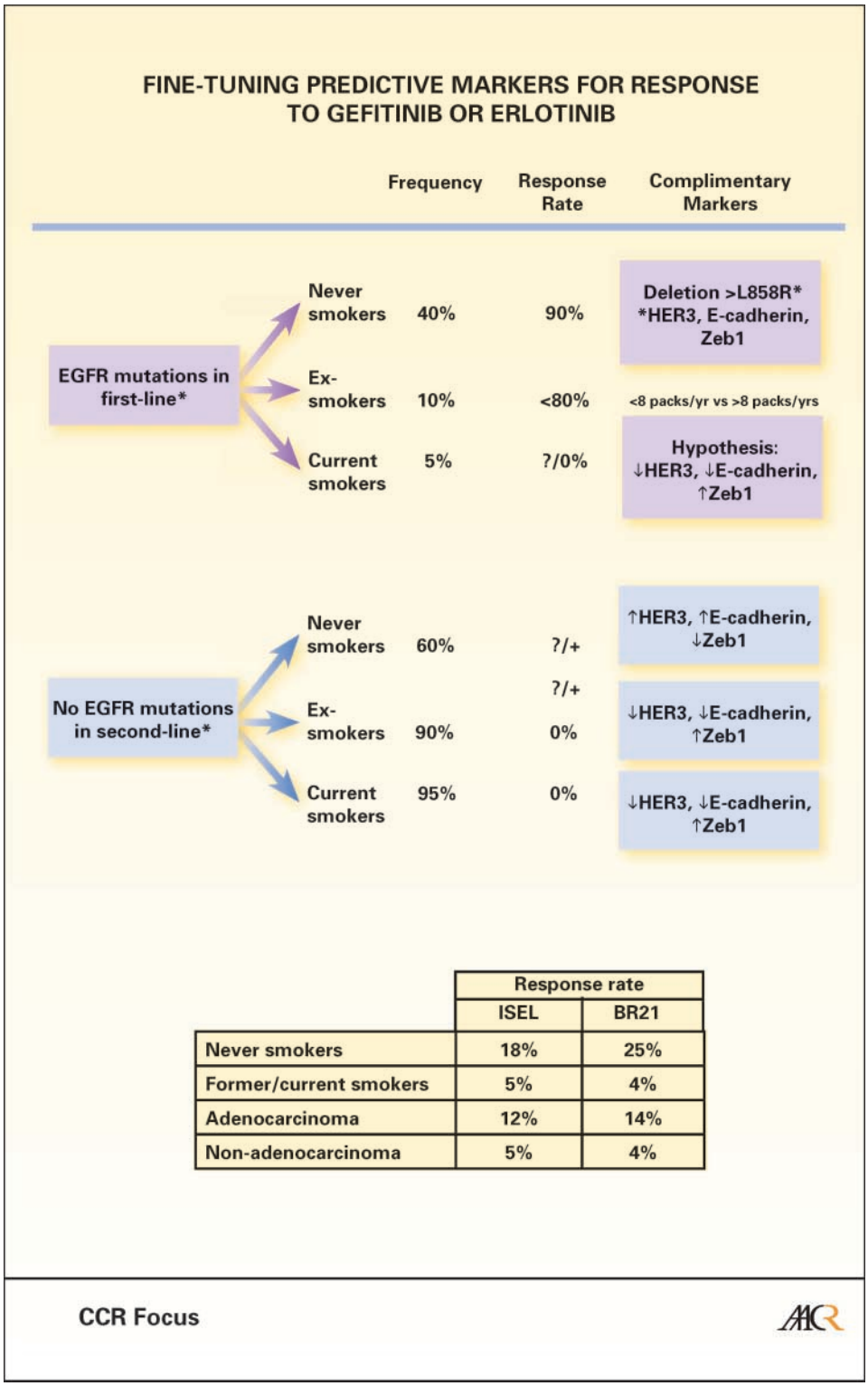
Considering a Role for HER2/HER3 in NSCLC

As noted earlier, the critical importance of EGFR mutations in conferring sensitivity to the tyrosine kinase inhibitors has raised the question of whether other mechanisms of EGFR activation exist, and highlighted the possibility that other approaches to EGFR inhibition may be required to address such mechanisms. Continuing with lung cancer as a model, the key questions are whether the EGFR tyrosine kinase inhibitors (gefitinib or erlotinib) are active in NSCLC patients without EGFR mutations and whether there are subsets of patients, such as never-smokers or ex-smokers without EGFR mutations, in whom overexpression of HER2 or HER3 can determine sensitivity to EGFR inhibitors, as has been shown in preclinical models (21, 29, 30). A predictive model (Fig. 3) might indicate that the benefit in clinical outcome in patients without EGFR mutations will be substantially less. Both HER2 and HER3 may need to be examined. Although it was initially found that increased *HER2* gene copy numbers were associated with better response, time to progression, and survival with gefitinib (31), a later study reported no differences in outcome either between patients with HER2- or HER3-positive tumors (by fluorescence *in situ* hybridization) and those with HER2- or HER3-negative tumors or according to phospho-AKT status (by immunohistochemistry; ref. 32). However, in cell lines, overexpression of HER2 and HER3 has been shown to confer gefitinib sensitivity, regardless of EGFR mutation status (21, 29). Ono and Kuwano (20) review the relevance of HER2/HER3-dependent cell signaling for gefitinib sensitivity in greater detail in this *CCR Focus*. The phosphorylation status of AKT (phospho-Akt) is one such marker that has been examined in 103 gefitinib-treated NSCLC patients, of whom 96 had received previous chemotherapy. Half were current smokers and only 19 were never-smokers. Positive phospho-Akt was associated with females, never-smokers, and bronchoalveolar histology. The response rate was better for patients whose tumors were positive for phospho-Akt (26.1% versus 3.9%; $P = 0.003$), as was the time to progression (5.5 versus 2.8 month; $P = 0.004$; ref. 33). In another series, the presence of positive phospho-Akt was not associated with response, although it was linked to longer time to progression (34).

[3] Unpublished data.

EGFR Copy Number and the Polymorphic CA Repeats

The question remains of whether EGFR overexpression can confer pathway activation in the absence of EGFR mutation, or HER2 or HER3 heterodimerization, and signaling. In some studies, gene amplification has been identified as a more important predictor of tyrosine kinase inhibitor benefit than EGFR mutations For example, Cappuzzo et al. (35) reported EGFR mutations in 17% of 89 Italian patients with NSCLC



	Response rate	
	ISEL	BR21
Never smokers	18%	25%
Former/current smokers	5%	4%
Adenocarcinoma	12%	14%
Non-adenocarcinoma	5%	4%

Fig. 3. Proposal for fine-tuning predictive markers for response to gefitinib or erlotinib based on our experience of response to erlotinib in NSCLC patients whose tumors harbor EGFR mutations. Top, EGFR mutations occur in 40% of never-smokers, with a 90% response rate, including complete responses. We hypothesize (*box*) that deletions could lead to better clinical outcome than L858R, as recently reported by Riely et al. (24), and that expression of HER3, E-cadherin, and Zeb-1 could be relevant ancillary factors in predicting clinical outcome. EGFR mutations are found in 10% of ex-smokers with a high response rate, but without complete responses. The number of pack-years could be relevant to survival in this subset of patients. The frequency of EGFR mutations is very low in current smokers, and preliminary data indicate that these patients do not respond to erlotinib. We hypothesize that HER3 and E-cadherin are down-regulated and Zeb-1 is up-regulated in these patients. Bottom, a common scenario where the vast majority of NSCLC patients do not have EGFR mutations. In all the studies of EGFR mutations, the response rate in patients with mutations ranges from 60% to 94%, whereas in patients without mutations, the response rate is 8% to 30%; time to progression is 12 to 21 and 1.7 to 3.6 mo, respectively. We hypothesize that never-smokers with wild-type EGFR could be relatively sensitive to EGFR tyrosine kinase inhibitors, as shown in cell lines. We hypothesize that EGFR-sensitive never-smokers will show high levels of HER3 and E-cadherin and low levels of Zeb-1. Based on clinical data, where response to erlotinib was 4% and to gefitinib 5% in NSCLC of former or current smokers, we predict that for the majority of ex-smokers without EGFR mutations, the chances of response will be practically nil. In the Thatcher et al. study (60), 60% of all patients were ex-smokers and only 17% were current smokers. The markers in the tumors (*box*) can help to explain the lack of response. Finally, for the majority of current smokers without EGFR mutations, the response rate to EGFR tyrosine kinase inhibitors is minimal. The table shows the response rates in the ISEL and BR.21 studies, broken down by smoking status and histology.

Table 2. Histology and EGFR mutations according to smoking status in NSCLC

Smoking status (*N* = 99)	*n* (%)	Histology			Mutation	
		Adeno	LCC	BAC	Del	L858R
Smoker	6 (6)	6	0	0	3	3
Ex-smoker	29 (29)	21	5	3	19	10
Never-smoker	64 (65)	52	5	6	40	24
		79	10	9	62	37
Total		98			99	

Abbreviations: Adeno, adenocarcinoma; LCC, large-cell carcinoma; BAC, bronchioloalveolar carcinoma; Del, deletion.

treated in second-line with gefitinib. Consistent with the findings of other investigators, patients whose tumors harbored a mutation had a better outcome. In addition, these investigators also analyzed *EGFR* gene copy numbers by fluorescence *in situ* hybridization in 102 patients and reported differences in favor of those with gene amplification in terms of response rate (36% versus 3%), time to progression (9 versus 2.5 months), and median survival (18.7 versus 7 months). However, there is no universal agreement on the sensitivity of fluorescence *in situ* hybridization assessment. In the Iressa Dose Evaluation in Advanced Lung Cancer trials, response to gefitinib was reported in 46% of patients with EGFR mutations, 29% of patients with increased gene copy numbers by fluorescence *in situ* hybridization, and in 9% of patients with neither mutations nor amplification ($P = 0.001$; ref. 36). In our experience (13), the response rate for patients with increased gene copy numbers was 45%, in contrast with 89% for patients with EGFR mutations ($P = 0.02$). It is possible that, in tumorigenesis, survival and selection for cells with the EGFR mutation concurrently select for cells that have amplification of the receptor.

Interestingly, in our study reported by Taron et al. (13), EGFR mutations were also associated with increased numbers of CA repeats in a polymorphic CA single sequence repeat in intron 1 of EGFR. Brandt et al. (37) review the importance of the CA single sequence repeats in intron 1 of EGFR in this *CCR Focus*. These repeats confer altered levels of *EGFR* gene transcription, with fewer repeats resulting in higher EGFR RNA and protein levels. Differences in these repeats have been observed in breast, colon, and head and neck cancer (37). Although increased numbers of repeats are thought to result in lower levels of EGFR expression, in Asian patients with breast cancer, any effect of the longer repeat seems to be overcome by *EGFR* amplification (38, 39). Whether this polymorphic sequence could confer differential expression that results in enhanced EGFR pathway activation (and perhaps sensitivity to cetuximab) in a subset of patients is not known. *In vitro*, cells with a lower number of CA repeats were more sensitive to erlotinib (40).

The Activating EGFRvIII Mutation in Glioma

Deletions of the extracellular domain of EGFR also have an activating effect on the receptor, providing cells expressing these truncated receptors a proliferative advantage. As reviewed by Nicholas et al. (41) in this *CCR Focus*, the most common truncated receptor is the variant III EGFR deletion mutant (EGFRvIII), which contains an in-frame deletion of exons 2 to 7 from the extracellular domain and is commonly observed in glioblastomas. EGFRvIII has recently been found to be present in a small percentage (5%) of human squamous cell lung cancers (42). Murine data confirm that overexpression of EGFRvIII is oncogenic in lung tissues. In addition, the abrogation of EGFRvIII expression by withholding doxycycline causes regression of the lung tumors, showing that these tumors are dependent on the activated EGFR pathway. It was also shown that HKI-272, an irreversible inhibitor that covalently binds to the EGFR kinase domain cleft, was effective in the treatment of EGFRvIII-dependent mouse lung tumors. Gefitinib and erlotinib also inhibit the growth of cells harboring EGFRvIII mutations, although at much higher concentrations than HKI-272. It is reasoned that this partial activity may provide an explanation for the reported response seen with erlotinib in a small percentage of squamous cell lung carcinomas (43). These findings suggest that HKI-272 could be effective for treating cancers that harbor the EGFRvIII mutations, including glioblastomas (42).

Complementary Markers Based on Epithelial-Mesenchymal Transition

Whereas examining the *EGFR* gene for mutations and downstream pathways for activation is to a certain extent intuitive, emerging data indicate that answers may also be found elsewhere. For example, a mesenchymal phenotype (44) has been linked to resistance to erlotinib in NSCLC cell lines and xenografts (45). Epithelial-mesenchymal transition (EMT) is a process whereby epithelial cell layers lose polarity and cell-cell contacts and undergo a dramatic remodeling of their cytoskeleton. Concurrent with a loss of epithelial cell adhesion and alterations in their cytoskeletal components, cells undergoing EMT acquire expression of mesenchymal components (44). A main feature of EMT is the loss of E-cadherin expression. In the TRIBUTE (Tarceva responses in conjunction with paclitaxel and carboplatin) trial (46), among patients receiving erlotinib and chemotherapy, time to progression was longer for those with E-cadherin-positive staining (47). Several important genes that induce EMT [*Snail, Twist, SIP1* (*Zeb-2*)] have been shown to act as E-cadherin repressors (48). Growth factors, including hepatocyte growth factor, transforming growth factor-β, and EGF, have also been found to induce EMT. Erlotinib-sensitive cell lines were E-cadherin positive and vimentin negative, whereas insensitive cell lines showed a pattern of EMT, with loss of E-cadherin and expression of vimentin or fibronectin (45). Interestingly, a cell line containing an EGFR deletion mutation (Δ746-750), sensitive to erlotinib, showed an E-cadherin-positive/vimentin-negative profile (45). Measurement of RNA abundance for *Snail, Twist, Zeb-1*, and *Zeb-2* (*SIP1*) showed that *Zeb-1* was the most highly expressed of the genes involved in the induction of EMT in erlotinib-insensitive NSCLC lines that had undergone EMT (45). In this regard, Witta et al. (49) have shown that pretreating resistant cell lines with a histone deacetylase inhibitor induces E-cadherin expression and leads to a growth-inhibitory and

apoptotic effect of gefitinib similar to that in gefitinib-sensitive cell lines, including those harboring EGFR mutations. Their rationale is that Zeb-1 inhibits E-cadherin expression by recruiting histone deacetylases. Whether this model can translate to the clinical setting remains to be seen (Fig. 3).

The EMT pattern has not been extensively studied in tumors with EGFR mutations. Both EGFR ligand and HER3 transcripts have been found to be elevated in erlotinib-sensitive NSCLC cell lines (47) and in gefitinib-sensitive human lung adenocarcinoma cell lines (50). The HCC2279 cell line, containing an EGFR deletion (Δ746-750) and undetectable levels of HER3, was only moderately sensitive to gefitinib (50). Together, these data suggest that the EMT phenotype and the HER3 mRNA levels could be good surrogate markers for response to EGFR inhibitors in patients with EGFR mutations (Fig. 3).

Cetuximab: Same Target, Different Mechanism of Action

The paradigm that EGFR mutations confer sensitivity to gefitinib or erlotinib provides no insight into whether these mutations also confer sensitivity to the monoclonal antibody cetuximab, and some data exist to suggest that they will not. Clinical responses to tyrosine kinase inhibitors after failure with cetuximab have been reported (51). Mechanistic differences between the two approaches to EGFR inhibition clearly exist (52): cetuximab binds to the cell-surface receptor, resulting in internalization and degradation, whereas the kinase inhibitors bind to the intracellular tyrosine kinase domain. Cetuximab may induce antibody-dependent cell-mediated cytotoxicity and thus may be able to target any cell-surface EGFR, whether mutated or not. Simple overepxression could confer sensitivity to cetuximab but not to the tyrosine kinase inhibitor. Treatment with gefitinib, erlotinib, or the anti-EGFR antibody cetuximab induced apoptosis in HCC827, a NSCLC cell line with *EGFR* gene amplification and exon 19 deletion (30). However, in other EGFR-mutant cell lines, cetuximab had relatively little effect (53). Intriguingly, in the gefitinib-insensitive H1975 cell line, which harbors two mutations (L858R and T790M), a dose-dependent induction of apoptosis was observed with cetuximab (47). This resistance-inducing secondary T790M EGFR mutation has been observed in half of drug-resistant NSCLCs, making the observation with cetuximab of particular relevance (11, 54, 55). Cetuximab also induced apoptosis in the EGFR-wild-type cell line expressing *K-ras* mutations (Hop18; ref. 47). Whereas the role of cetuximab in NSCLC is still not completely defined, it is well established in colorectal cancer, in which *EGFR* amplification and the absence of *K-ras* mutations are associated with cetuximab response (56, 57).

Conclusion

A growing body of evidence confirms the high response rate and impressive survival benefit of EGFR tyrosine kinase inhibitors in patients harboring EGFR mutations (13, 16, 58, 59). This is in marked contrast with the clinical outcome attained with indiscriminate use of gefitinib or erlotinib, as observed in the BR.21 trial (7, 43) and the Iressa Survival Evaluation in Lung Cancer (ISEL; ref. 60) studies (Fig. 3). Whereas the indiscriminate use of these agents can no longer be recommended, the various mechanisms of EGFR activation discovered during investigation of this molecular target suggest that other approaches to inhibition of the pathway may yet be successful. Meanwhile, a valuable tool has been added to our chemotherapy arsenal for a subset of patients. More importantly, as we more precisely understand mechanisms of EGFR pathway activation, we have learned that patience is required to dissect the pathway, to understand its contribution to the malignant phenotype in the clinical setting, and to develop it as a therapeutic target.

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