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Review

EGFR Mutations in Non-small Cell Lung Cancer – Clinical Implications

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Abstract. *The epidermal growth factor receptor (EGFR) family members seem to play a critical role in lung tumourigenesis and are overexpressed in 40-80% of non-small cell lung carcinoma (NSCLC) tumours. EGFR activation results in a series of downstream signaling events that mediate cancer cell growth, proliferation, motility, adhesion, invasion, apoptosis inhibition and metastasis as well as resistance to chemotherapy. Therefore, EGFR inhibitors seem to be an effective therapy for some patients with previously treated NSCLC. A thorough investigation of EGFR, its major signaling pathways, its identification and biology in NSCLC and the responsiveness to gefitinib, erlotinib and cetuximab in connection to EGFR mutations as well as the possible mechanisms of resistance to tyrosine kinase inhibitors is the scope of this review.*

Lung cancer displays a great heterogeneity in clinical behavior and seems to share a limited set of acquired capabilities such as insensitivity in growth signals, unlimited replicative potential, tissue invasion, sustained angiogenesis and metastasis (1-3). Among these, the acquisition of unlimited replicative potential is a key step to ensure expansive tumour growth. The epidermal growth factor receptor (EGFR) family members seem to play a critical role in lung tumourigenesis and are overexpressed in 40-80% of non-small cell lung carcinoma (NSCLC) (4). EGFR activation results in a series of downstream signaling events that mediate cancer cell growth, proliferation, motility, adhesion, invasion, apoptosis inhibition and metastasis as well as resistance to chemotherapy (5-7). Therefore, EGFR inhibitors seem to be an effective therapy for some patients with previously treated NSCLC. The scope of this review is to investigate EGFR, its major signaling pathways, its identification and biology in NSCLC and the responsiveness to gefitinib, erlotinib and cetuximab in connection to *EGFR* mutations as well as the possible mechanisms of resistance to tyrosine kinase inhibitors.

Abbreviations: EGFR: epidermal growth factor receptor; NSCLC: non-small cell lung carcinoma; TK: tyrosine kinase; ATP: adenosine triphosphate; MAPK: mitogen-activated protein kinase; PI3K: phosphatidylinositol-3-kinase; STAT: signal transducer and activator of transcription proteins; EGF: epidermal growth factor; TGFα: tumour growth factor-α; TKI: tyrosine kinase inhibitor; IDEAL: Iressa® Dose Evaluation in Advanced Lung Cancer; INTACT: Iressa® NSCLC Trials Assessing Combination Therapy.

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What is EGFR?

EGFR is a 170 kDa membrane bound protein encoded by 28 exons on chromosome 7p12. It is a typical member of the tyrosine kinase family (TK) and belongs to a subfamily that consists of four closely related members: EGFR (ErbB1), HER-2/neu (ErbB2), HER-3 (ErbB3) and HER-4 (ErbB4). All members have an extracellular ligand-binding, a single membrane spanning and an intracellular domain (8, 9) and are known to have intrinsic TK activity except for ErbB3 (10). Ligand binding results in homo- or heterodimerisation, activation of their highly conserved intracellular kinase domain and autophosphorylation of tyrosine residues by γ-phosphates from adenosine triphosphate (ATP). The latter serve as docking sites of proteins, whose recruitment activate downstream signaling pathways (3, 8, 11, 12).

Major signaling pathways activated by EGFR. ErbB specific binding to effector proteins results in ErbB activation and stimulation of several intracellular signaling pathways. The

main pathways activated by the ErbB family are two: the mitogen-activated protein kinase (MAPK) cascade, which induces proliferation and the phosphatidylinositol 3-kinase [(PI3K)-AKT] and the janus kinase (JAKs) and signal transducer and activator of transcription proteins (STATs) pathways, which play an important role in cell survival (9, 13) (Figure 1). In human cancer cells, the EGFR pathway becomes hyperactivated mainly by three mechanisms: overproduction of ligands, such as epidermal growth factor (EGF) and tumour growth factor-α (TGFα), *EGFR* gene amplification and mutational activation of EGFR. EGFR seems to play a crucial role in carcinogenesis, since EGFR pathway activation due to mutations (14), gene amplifications (15, 16), or ligand and/or receptor overexpression (17) has been found in several cancer types, in comparison to normal tissues. Such observations led to the conclusion that *EGFR* gene is an oncogene and targeting the receptor might be a successful way to treat EGFR-expressing cancers.

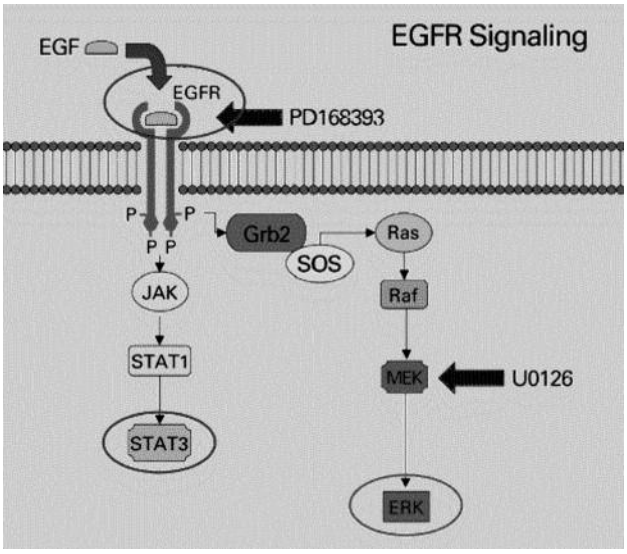

Figure 1. *A principal pathway for mitogen-activated kinase (MAPK) activation by EGF consists of sequential activations of the guanine nucleotide exchange factor (SOS), the guanosine triphosphate-binding protein Ras, and the protein kinases Raf-1, MAPK kinase (MKK), and MAPK. In mammals, the janus kinase and signal transducer and activator of transcription proteins (JAK/STAT) pathway is the principal signaling mechanism for a wide array of cytokines and growth factors. JAK activation stimulates cell proliferation, differentiation, cell migration and apoptosis. These cellular events are critical to hematopoiesis, immune development, mammary gland development and lactation, adipogenesis, sexually dimorphic growth and other processes. Two receptor subunits must be associated with JAK tyrosine kinases. JAKs are distinctive in that they have tandem kinase-homologous domains at the C-terminus. The first is a non-catalytic regulatory domain, whereas the second has tyrosine kinase activity. In mammals, the JAK family comprises four members: JAK1, JAK2, JAK 3 and Tyk2. JAK activation occurs upon ligand-mediated receptor multimerization because two JAKs are brought into close proximity, allowing trans-phosphorylation. The activated JAKs subsequently phosphorylate additional targets, including both the receptors and the major substrates, STATs. STATs are latent transcription factors that reside in the cytoplasm until activated.*

Identification of EGFR Mutations in NSCLC Tumours

In many tumours EGF-related growth factors are produced by the tumour cells themselves or are available from surrounding stromal cells and result in EGFR activation. The approaches that led to the identification of EGFR TK domain mutations in NSCLC patients were based on the hypothesis, that patients presenting striking clinical responses to gefitinib, a small molecule tyrosine kinase inhibitor (TKI), had somatic mutations in EGFR (18). Lynch and colleagues (18) sequenced the entire coding region of the *EGFR* gene in patients presenting a clinical response to gefitinib and Paez and colleagues (19) subsequently performed a genome-wide screening of the TK receptors. *EGFR* mutations have been identified in both approaches, more frequently in adenocarcinomas (particularly bronchioalveolar carcinomas), women, non-smokers and patients of East Asian origin (18-20). These patients were most likely to obtain tumour shrinkage from EGFR TKIs. Further studies have collectively detected 192 *EGFR* mutations in NSCLC tumour specimens (18, 19, 21).

Types of EGFR mutations detected in NSCLC. Tyrosine kinase domains are highly conserved and consist of two approximately globular structures, a large C- and a smaller N-lobe. Activating mutations (point mutations, deletions and insertions) in the TK domain of *EGFR* are limited to exons 18, 19, 21 and 24 and show a great structural diversity (12, 18, 19, 22-24). The majority of the *EGFR* mutations identified are heterozygous, implying that they are dominant, are present only in the tumour and, to date, no germline mutations have been described. *EGFR* mutations seem to cluster around the ATP-binding pocket of the TK domain and result in alteration of residues when in contact with ATP, which in turn leads to alteration of the regulation of the kinase. Overlapping deletions in exon 19 and point mutations in exon 21, account for about 85% of all mutations (18, 19, 21). All NSCLC-specific *EGFR* mutations are clustered within the kinase domain of EGFR and can result in increased activity of the receptor. However, only the G719C (exon 18), E746-A450 deletions in exon 19, L858R (exon 21), L861Q (exon 21) and R776C (exon 20) mutations are related with a clinical response to TKIs (18).

Biology of EGFR mutant NSCLC. The identification of *EGFR* mutations revealed that *EGFR* mutant NSCLC tumours and cell lines are biologically distinct from the other

NSCLC. It has been suggested that the mutant EGFR protein differs from the wild-type (18). Non-small cell lung cancer cell lines that contain *EGFR* mutations undergo extensive apoptosis when treated either with AKT/STAT inhibitors or after short interfering RNA-mediated knockdown of the mutant *EGFR* (25). On the contrary, NSCLC cell lines that harbor the wild-type EGFR do not seem to be affected by the same treatments. Although many experiments have been performed, the specific differences in EGFR-dependent signaling between the wild-type and mutant EGFR still remain poorly understood (19, 24). Interestingly enough, 40% of the NSCLC tumours that harbor *EGFR* mutations are also characterized by amplification of the mutant gene (21). *EGFR* gene amplification, in general, has been detected in 9% of NSCLC tumours and it has been suggested that the amplification itself and/or in conjunction with TK domain mutations is related to sensitivity to small TKIs (26).

EGFR Mutations in Unselected Patients Responding to TKIs

EGFR associated tyrosine kinase inhibitors represent a novel approach for the treatment of solid tumours by interfering with cancer cell signaling pathways. At least four ErbB-targeted TKIs have been evaluated: gefitinib, erlotinib, lapanitib and carnetinib. All compounds share a common 4-anilinoquinazoline core in which the quinazoline ring is hydrogen bonded to the region between NH_2- and COOH-terminal of the kinase. However, they seem to have a distinct ErbB inhibition profile and mechanism of action (27, 28). Gefitinib and erlotinib are potent selective inhibitors of EGFR, while lapatinib is inhibitor of both EGFR and ErbB2. Carnetinib covalently modifies an active-site cysteine from a template with high initial EGFR activity (41). Somatic TK mutations of the *EGFR* gene increase the sensitivity of NSCLC tumours to TKIs, by repositioning critical residues near the ATP-binding cleft of the TK domain of the receptor. The interactions of the latter with both ATP and its competitive inhibitors are thus stabilized (18, 19). EGFR TKIs have shown their most promising clinical activity as monotherapy in patients with previously treated advanced NSCLC (18, 19, 29, 30). However, when used in combination with chemotherapy in first line, no additional benefit was provided as measured by response rate, with either gefitinib or erlotinib (31-34). Interestingly, 18% of patients with wild-type EGFR NSCLC tumours who received TKIs achieved a partial response (24). A possible explanation may be that some mutations were missed by sequencing. Alternatively, there may be other molecular determinants of the tumour sensitivity to TKIs, such as HER-2/neu. Mutations in HER-2/neu have been identified in 10% of lung adenocarcinomas (35), however in none of the patients receiving TKIs. A third possibility is that there may be lung cancer types whose growth and metastatic potential depends on wild type EGFR signaling and are thus very sensitive to TKIs.

Gefitinib a small molecule EGFR TKI. Gefitinib is an orally available synthetic anilinoquinazoline agent which selectively binds to the TK region of the intracellular domain of EGFR, prevents ATP binding and blocks EGFR signalling transduction pathways (36). In two multicenter phase II trials: the Iressa® Dose Evaluation in Advanced Lung Cancer (IDEAL-1) in Japan and Europe with 210 patients enrolled and the IDEAL-2 trial in the United States including 221 patients (37, 38) the results indicated that gefitinib demonstrated encouraging anti tumour activity and was well tolerated in pretreated advanced NSCLC patients. The overall response rate was 18% in the IDEAL-1 and 10% in the IDEAL-2 trial and symptom improvement in 40% of symptomatic patients. Based on these results, gefitinib received approval as monotherapy in patients with locally advanced or metastatic NSCLC after failure of both platinum-based and docetaxel chemotherapies (36). The recommended dose was 250 mg administered daily, as no additional benefit has been detected with treatment at higher doses. Based on the additive and synergistic activity of gefitinib with cytotoxic chemotherapy, two multinational randomised double-blind placebo-controlled phase III studies compared standard chemotherapy with chemotherapy and two different doses of gefitinib. One thousand and ninety three chemotherapy-naïve NSCLC patients were assigned to gemcitabine-cisplatin chemotherapy plus placebo or gefitinib in Iressa® NSCLC Trials Assessing Combination Therapy (INTACT-1) (33). In the INTACT-2 study, 1037 NSCLC patients were allocated to carboplatin/paclitaxel chemotherapy plus placebo or gefitinib (32). Both studies failed to confirm any benefit in survival, time to progression, or response rate for patients in the gefitinib arm. Molecular analyses of the IDEAL and INTACT trials were subsequently performed on the basis of presence of *EGFR* mutations and/or *EGFR* gene amplification. For the IDEAL-1 and -2 study, 119 tumour specimens (28% of all cases) were available for molecular analysis and *EGFR* mutations were found in 13 out of 79 cases (17%) successfully sequenced (39). Objective responses to gefitinib were achieved in 6 of 13 tumours (46%) harbouring an *EGFR* mutation, 2 of 7 tumours (29%) with *EGFR* gene amplification and 5 of 56 tumours (9%) with neither mutation nor amplification. However, an improved overall survival was not evident in patients with *EGFR* mutant tumours (39). In the INTACT studies, EGFR mutations were detected in 32 of 312 cases (10%) and the statistical analysis did not reveal a significant difference in response to gefitinib plus chemotherapy according to *EGFR* genotype. The INTACT studies, however, revealed a significantly increased survival of patients with

EGFR mutant NSCLC tumours, irrespective of gefitinib treatment and a similar trend in patients with *EGFR* gene amplification. The data above imply that these tumour subtypes have a distinct natural history and their contribution to enhanced survival has to be confirmed in population based studies (39). Moreover a subgroup analysis of 136 NSCLC patients also suggested that non-smokers have a three-fold higher objective response rate than smokers (40).

Is responsiveness to gefitinib dependent on EGFR gene ampification? Amplification of the *EGFR* locus has been detected in 8% and 7% of cases in the IDEAL and INTACT studies, respectively. No correlation has been found between EGFR amplification and response to gefitinib, even in cases with clinical features that are characteristic of strong responses to the latter (39). *EGFR* amplification accounted for a small number of cases with high levels of protein expression as measured by immunohistochemistry. The mutational status of EGFR was available in 14 cases with EGFR gene amplification and, interestingly enough, 10 cases had amplification of the wild-type *EGFR*. The role, however, of the amplification of the wild-type *EGFR* as an independent predictor of gefitinib response has to be further investigated. Taken together, molecular analysis of the IDEAL studies revealed that 60% of cases with genetic lesions in *EGFR* (mutations or gene amplification) responded to gefitinib, compared to 10% of those without either genetic abnormality.

Is responsiveness to gefitinib dependent on EGFR protein expression? Despite the fact that a high expression level of EGFR has been associated with lower relapse-free and overall survival rates in a range of malignancies, sensitivity to gefitinib does not seem to be correlated with the receptor's overexpression. An exploratory analysis of tumour biopsies taken from patients of the IDEAL-1 and-2 trials has been performed in order to correlate the immunohistochemical expression of EGFR with tumour response or symptom improvement (39). The results of the analysis did not reveal an association between EGFR membrane staining (as assessed from 0 to +3) and either objective response or symptom improvement. On the contrary, several EGFR-negative patients benefited from gefitinib, whereas some patients with intense EGFR staining did not show any response (41, 42). Based on these data, it seems that inhibition of EGFR autophosphorylation rather than overexpression of the receptor results in the activity of gefitinib.

Erlotinib. Erlotinib is a reversible, ATP-competitive inhibitor of the wild-type EGFR TK. Erlotinib has a small aniline substitution off the quinazoline ring and this conformation is nearly identical to apo-EGFR. This results in binding and dissociating of the inhibitor from the enzyme's active form without requiring any major changes in protein conformation (27).

Preclinical studies have shown that erlotinib inhibits cell cycle progression and Rb phosphorylation, while it induces p27 expression and apoptosis (43). An overall response rate of 12.3% was detected in a phase II trial involving 57 NSCLC patients previously treated with platinum-based chemotherapy (44). Interestingly enough, patients with bronchioalveolar carcinoma (BAC) showed a response rate of 25% (45). Three phase III trials have been performed in order to test the efficacy of erlotinib given concurrently with chemotherapy. A total of 1,059 (TRIBUTE) and 1,172 (TALENT) chemotherapy-naïve NSCLC patients were allocated to carboplatin/paclitaxel and cisplatin/gemcitabine plus placebo or erlotinib respectively (31, 33, 34). Both studies indicated that erlotinib combined with standard chemotherapy did not confer a survival advantage over chemotherapy alone in patients with previously untreated advanced NSCLC. Tumour specimens were available for *EGFR* sequencing in 228 patients of the TRIBUTE study (46) and mutations were found in 29 (12.7%) of them. The response rate to chemotherapy alone was similar between carriers of mutant and wild-type *EGFR*. However, subgroup analysis demonstrated that there was a significant better clinical outcome in patients carrying the mutant *EGFR* than the wild-type one in all assessed end points (46). In the same study, mutational analysis of *K-ras* revealed that *K-ras* mutations were associated with significantly lower time to progression and survival in the subgroup receiving erlotinib plus standard chemotherapy (46). In a phase III randomized trial (BR.21), erlotinib was compared with placebo in 731 NSCLC patients who had failed prior chemotherapy and a correlation to survival benefit was detected (29). There was a 42.5% improvement in median survival (4.7 *versus* 6.7 months) and a 41% improvement in one-year survival rates (21% *versus* 31%). This was the first study that proved that an EGFR TKI presents a survival benefit in NSCLC patients after first- or second-line standard chemotherapy. In the BR.21 study, *EGFR* mutations appeared to have a lower predictive value in the response to erlotinib (47). Univariate analysis from 427 patients showed that *EGFR* polysomy, or amplification, as well as EGFR protein as measured by immunohistochemistry were significantly associated with responsiveness to erlotinib. In multivariate analysis, however, survival after treatment with erlotinib was not influenced by EGFR protein expression, gene copies or mutations. The data above suggest that *EGFR* mutations may increase responsiveness to erlotinib, but are not correlated to a survival benefit (47).

Do EGFR mutation determine responsiveness of NSCL to EGFR-specific antibodies? It is currently unknown whether *EGFR* mutations predict sensitivity to treatment with an

EGFR-directed antibody. Tsuchihashi and colleagues sequenced the kinase domain of EGFR in tumour samples from 38 patients receiving cetuximab monotherapy for recurrent NSCLC (48). None of the previously detected mutations was identified in NSCLC patients who had a partial response, while two patients with stable disease carried a del746-750 and one patient with disease progression carried an L861Q mutation (48). These data, despite the fact that they are based on small number patients, suggest that *EGFR* mutations are not related with sensitivity to cetuximab. However, further research is needed in this field.

Resistance to TKIs

Despite the fact that *EGFR* mutations confer sensitivity of NSCLC to anilinoquinazoline inhibitors, most patients eventually progress.

Primary resistance to TKIs: Can K-RAS mutational status predict the clinical outcome? K-ras mutations have been associated with some cases of primary resistance to gefitinib and erlotinib (49). *K-ras* is an EGFR downstream signaling molecule and activating mutations have been identified in up to 30% of lung adenocarcinomas. They are more common in women and seem to be related to a history of cigarette smoking (50). *K-ras* and *EGFR* mutations have been found to be mutually exclusive (21); a fact that may reflect their equivalent role in NSCLC tumourigenesis. Eberhard and colleagues also stated that patients carrying *K-ras* mutations not only failed to benefit from erlotinib plus chemotherapy, but also suffered a worse prognosis compared to those receiving chemotherapy alone (46). The data above suggest that *K-ras* mutations may be a predictor of response to TKIs and that *K-ras* mutational status should also be identified prior to treatment with a small molecule TKI.

How does EGFR mutant NSCLC become resistant to TKIs? Acquired resistance to TKIs. Despite the clinical benefit acquired in NSCLC carriers of *EGFR* mutations when treated with small molecule TKIs, a large number of these patients will eventually develop disease progression. The underlying mechanism of this acquired or secondary resistance is still poorly understood. There are two studies that examined NSCLC tumour specimens from patients with *EGFR* mutations, treated either with gefitinib or erlotinib, who initially demonstrated partial or complete responses but finally suffered progressive disease (51, 52). A substitution of methionine for threonine at position 790 (T790M) in exon 20 was identified in 4 out of 7 tumour specimens analyzed. This mutation has not been detected in specimens from untreated patients, while none of the tumours with the secondary mutation harbored K-ras mutations. Tumour subclones carrying this mutation could arise *de novo* during treatment, or most possibly pre-exist at levels below the threshold of detection within the primary tumour clone (52, 53). Either way, treatment with a small molecule TKI allows these subclones to become apparent because the cells bearing *EGFR* mutations die, while those with the T790M mutation survive. Second generation EGFR inhibitors need to be developed that bind to the TK domain in different ways than gefitinib and erlotinib. Wood and colleagues examined the crystal structure of lapatinib and concluded that the quinazoline rings of erlotinib and lapatinib interact differently with the TK domain and that the T790M mutation may not affect inhibition of EGFR by compounds similar to lapatinib (27). However, other genes or molecular pathways may be involved in acquired resistance to TKIs, as suggested by the existence of a number of TKI recurrent patients without a secondary mutation. Therefore, inhibitors of downstream targets of EGFR such as PI3K or STAT5 may also be of use for patients with *EGFR* mutant tumours and acquired resistance to TKIs.

Conclusion

Even though *EGFR* mutations are associated with sensitivity to gefitinib and erlotinib as discussed above, they do not account for all the clinical benefit observed in all NSCLC patients receiving gefitinib or erlotinib. Lynch and Paez showed that 6/31 (~20%) of the patients that experienced partial responses or clinical improvement were carriers of the wild-type EGFR. It was thus suggested that there might be other mechanisms involving wild-type EGFR resulting in drug sensitivity. Most of the applied clinical research on EGFR has been focused on the overexpression of the receptor, whereas less research has addressed the potential role of other mechanisms of increased signalling, as well as non-membrane bound events in the modulation of specific biological behaviours. Furthermore, to date, there is no data on clinical differences as measured by response rate, time to progression or survival in patients with different types of *EGFR* mutations treated with EGFR TKIs. The prognostic significance of EGFR expression in lung cancer and, more importantly, its ability to predict response to anti-EGFR therapies, are currently subjects of active research. The current data suggest that advanced NSCLC chemotherapy-naïve patients should undergo *EGFR* sequencing before receiving treatment. Therapeutic options may thus be guided towards TKIs in patients harbouring *EGFR* mutations. The effect of TKIs should be examined in specific molecular and clinical subgroups of NSCLC in order to identify the most effective target therapy. New technologies such as nucleotide arrays and proteomics will help to elucidate this issue by providing information on how EGFR signalling may affect the expression of genes and proteins in cancer cells. The great majority of data on *EGFR* mutations were acquired from retrospective studies and subjected to selection biases. All the findings need to be validated prospectively and studies that

prospectively validate the correlation between *EGFR* mutations and the response to TKIs are currently underway. The impact of EGFR mutation screening on the design of first-line studies for NSCLC patients remains to be defined. Future studies may select patient groups most likely to benefit from TKIs (women, nonsmokers, adenocarcinomas) and further selection criteria may be the presence of *EGFR* and absence of *K-ras* mutations. Several questions remain to be addressed in future studies. Should TKIs be used in the adjuvant and neoadjuvant setting as therapeutic options in NSCLC patients that carry *EGFR* mutations? Should chemotherapy-naïve NSCLC patients with EGFR mutations receive TKIs as first line treatment? What is the optimal treatment for patients that carry *K-ras* mutations or presenting acquired resistance to TKIs? To date, available data suggest that *EGFR* mutations are associated with a greater likelihood of objective response to EGFR TKI inhibitors. However, it is not yet known whether patients with *EGFR* mutant NSCLC tumours gain a survival benefit when treated with EGFR inhibitors, or if the mutations are positive prognostic indicators outside the setting of EGFR inhibition.

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