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Review

Epidermal growth factor receptor (EGFR) as a target in cancer therapy: understanding the role of receptor expression and other molecular determinants that could influence the response to anti-EGFR drugs

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Abstract

The epidermal growth factor receptor (EGFR) is a rational target for cancer therapy because it is commonly expressed at a high level in a variety of solid tumours and it has been implicated in the control of cell survival, proliferation, metastasis and angiogenesis. However, despite evidence to suggest that EGFR expression is associated with a poor prognosis in some tumours (e.g. breast, head and neck carcinomas), the situation is by no means clear-cut. A number of issues are worthy of particular consideration, including how EGFR is measured and whether these assays are sensitive and reproducible, which mechanisms other than increased EGFR expression might cause the EGFR signalling drive to be increased, and the relationship, if any, between EGFR expression and the response to EGFR-targeted agents.

Keywords: Epidermal growth factor receptor (EGFR); EGFR-targeted therapy; Signalling pathways; EGFR-TKI; Gefitinib ('Iressa', ZD1839)

1. Introduction

Several lines of evidence have identified the epidermal growth factor receptor (EGFR) as a rational target for anticancer therapy [1,2]. The receptor is expressed or highly expressed in a range of solid tumours including breast, head and neck, non-small cell lung and prostate cancer [3]; for example, in an analysis of >5000 patients with breast cancer, the mean percentage of EGFR positivity was 45% (range 14–91%) [4]. In some tumours, EGFR has been associated with an advanced tumour stage [3], poor prognosis, and resistance to chemotherapy, hormone therapy and radiation [5,6]. EGFR (erbB1) is one of a family of four erbB receptors that have a common structure comprising an extracellular ligand-binding domain, a transmembrane domain and an intracellular domain with tyrosine kinase activity for signal transduction. Binding of a ligand such as the epidermal growth factor (EGF) or transforming growth factor α (TGFα) causes EGFR to dimerise or to heterodimerise with another member of the erbB family. This leads to receptor-linked tyrosine kinase activation and results in a signalling cascade that produces diverse effects including cell migration, maturation, differentiation, metastasis, angiogenesis and inhibition of apoptosis [7,8].

A range of EGFR-targeted agents are in development, including those directed at the receptor's extracellular domain, such as monoclonal antibodies [9], and those with an intracellular site of action, such as small-molecule tyrosine kinase inhibitors [10–12]. Preclinical and early clinical data from such compounds have been encouraging; however, there are several key issues that must be considered if these agents are to reach their maximum potential. At a practical level, there is no universal method for evaluating EGFR expression and

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the relationship between expression level and prognosis is far from clear. Of particular interest in the clinical setting is whether EGFR expression levels can predict the response to therapy. Receptor expression cannot be assumed to predict response because the EGFR signalling network is comprised of a complex series of interconnecting pathways and each component is likely to affect the level of EGFR signalling output.

2. Evaluation of EGFR

EGFR can be evaluated in different ways: quantitation of the receptor at the DNA, RNA or protein level, or assessment of the degree of signalling from the receptor through analysis of receptor activation or activation of downstream markers.

Analysis of DNA will provide information on amplification, deletion or mutation of the EGFR gene. However, this does not necessarily reflect the amount of protein produced. Northern blotting or quantitative reverse transcription polymerase chain reaction (PCR) can be used to assess mRNA levels, although techniques based on mRNA are associated with problems of RNA degradation and contamination. Posttranslational modifications might affect the quantity of protein produced; therefore, it may not be appropriate to make assumptions about EGFR levels based on mRNA measurements.

Immunohistochemistry (IHC) is commonly used to evaluate EGFR protein levels and is arguably the most convenient method for analysing clinical samples [13]. Advantages of the technique include the ability to determine the subcellular localisation of protein, to make comparisons between samples and to evaluate the levels of activated (phosphorylated) EGFR. However, this method is not strictly quantitative as there is no uniform scoring system and the interpretation of staining intensity is highly subjective. In general, samples are scored as EGFR-positive when either the percentage of stained cells or the staining intensity is above a specified threshold level and are considered EGFR-negative when below this threshold. In addition, variations in protocols, such as in fixation procedures and antibodies, are likely to affect the sensitivity of these assays, making comparison of results from different laboratories difficult. Radioimmunohistochemistry has been found to be more quantitative than standard IHC, as demonstrated in a study by Robertson and colleagues on frozen tissue sections from 203 primary breast carcinoma specimens [14].

EGFR protein levels may also be quantified by western blot analysis and enzyme immunoassay. These methods measure total receptor protein and provide no information on cellular localisation. EGF binding assays with radioactive ^{125}I-EGF use autoradiography to determine the localisation of accessible, unoccupied cell surface receptors, but considerable interassay variability has been reported [14].

Activation of the EGFR signalling network can be estimated by measurement of EGFR phosphorylation using, for example, IHC or immunoprecipitation, or assessment of downstream targets of EGFR, such as proliferation and maturation markers [15,16].

3. EGFR and prognosis

The relationship between EGFR expression and cancer prognosis has been investigated in many studies in a range of tumours, often with discrepancies in the reported results. For example, Nicholson and colleagues carried out a retrospective analysis of over 200 studies and found that EGFR acted as a strong prognostic indicator in some cancers, including head and neck, ovarian and cervical, but only as a modest or weak indicator in others, such as endometrial and non-small cell lung cancers [13]. The authors noted that the studies were not standardised with respect to the patient populations and the assays used to determine tumour EGFR levels, and also that failure to identify EGFR as a prognostic indicator did not necessarily mean that patients would not benefit from EGFR-targeted therapies. The lack of a clear picture with regard to the relationship between EGFR and prognosis is highlighted by the fact that other studies have shown that EGFR does appear to have prognostic value in patients with lung cancer [17].

Two recent studies involving immunohistochemical analysis of EGFR expression in breast tumours have both shown a significant correlation of EGFR positivity with a shorter disease-free and overall survival [18,19]. Aziz and colleagues analysed 315 tumour specimens and found high expression of EGFR in 70 cases (22%) [18]. At a median follow-up of 48 months, EGFR-positive patients had a shorter overall survival than EGFR-negative patients (3.39 years versus 4.62 years, respectively). Similarly, disease-free survival was shorter in EGFR-positive patients (2.86 years versus 4.00 years, respectively). In a large, prospective study, Tsutsui and colleagues evaluated 1029 patients with breast cancer who had undergone a surgical operation at a single centre [19]. EGFR positivity in tumour cells was observed in 277 cases (27%) and univariate analysis showed a significantly worse clinical outcome for EGFR-positive patients compared with EGFR-negative patients for both overall ($P<0.0001$) and disease-free survival ($P<0.0001$) (Fig. 1).

EGFR determination by radiolabelled EGF binding was also found to be a powerful prognostic parameter in a study of 77 patients with unresectable pharyngeal cancer treated with concomitant chemoradiotherapy

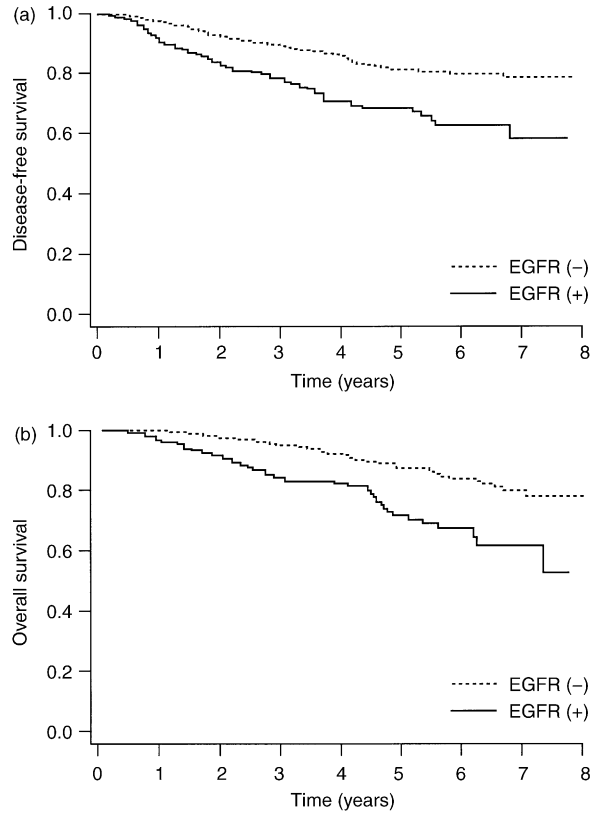

Fig. 1. Disease-free (a) and overall survival (b) curves for patients with breast cancer according to epidermal growth factor receptor (EGFR) [19]. Reproduced with permission from Kluwer Academic Publishers.

[20]. When patients were stratified on the basis of tumour EGFR levels, those with <35, 35–275 and >275 fmol/mg protein had 3-year overall survival rates of 95, 51 and 16%, respectively.

It is apparent that no consensus has been reached on the role of EGFR as a prognostic indicator in solid tumours, partly due to studies using a small sample size and a range of different assays. In the future, larger studies that employ a standard assay should yield more information.

4. Enhancement of EGFR-driven intracellular signalling

High expression of EGFR is commonly thought of as the main mechanism by which EGFR signalling is increased in cancer cells. However, a number of alternative mechanisms are likely to be of importance (Fig. 2), including activating EGFR mutations, decreased levels of phosphatase, increased coexpression of receptor ligands, such as transforming growth factor α (TGFα) and amphiregulin, and heterodimerisation with HER2 and/or the other members of the erbB receptor family, as well as interaction with heterologous receptor systems. Some of these mechanisms have even been found to have an impact on prognosis.

4.1. EGFR mutations

The best-described and most common EGFR mutation, EGFRvIII, lacks part of the extracellular ligand-binding domain due to deletion of exons 2–7, which results in a constitutively active receptor that is not downregulated by endocytosis and is potently transforming [21]. This variant is frequently expressed in various tumour types, including glial, breast, ovarian and non-small cell lung tumours [22]. It is expected that EGFR antibodies raised against extracellular receptor epitopes deleted in this mutant would not have activity in this setting, whereas small-molecule tyrosine kinase inhibitors might have activity, as has been demonstrated by inhibition of autophosphorylation of EGFRvIII in NR6M cells in culture [23].

4.2. Increased ligand expression and enhanced autocrine loop

Increased levels of EGFR-specific ligands such as EGF, amphiregulin or TGFα will have an impact on intracellular signalling. Evidence to support the existence of an autocrine loop comes from the observation that most epithelial cancers that express high levels of TGFα coexpress EGFR [3,24]. For example, in a study of 173 patients with invasive ductal breast carcinoma, a multivariate analysis found that coexpression of EGFR and TGFα had the most significant effect on overall ($P<0.0001$) and disease-free ($P<0.0001$) survival compared with, for example, oestrogen receptor expression [25]. Immunohistochemical analysis of 131 primary human lung adenocarcinomas showed that, for EGFR-positive cases, 5-year survival rates were significantly worse for patients with high levels of expression of EGF or TGFα compared with those who had low expression of EGF or TGFα [26]. In a study of 91 patients with head and neck cancers that were treated by radical surgical resection, overexpression of both EGFR and TGFα significantly ($P<0.0001$) predicted a shorter disease-free and overall survival in a multivariate analysis [27].

4.3. Heterodimerisation and cross-talk among different erbB receptor family members

EGFR signalling is also dependent upon the differential formation of receptor dimers. Each erbB receptor has some different characteristics: erbB3 has no intrinsic tyrosine kinase activity; a direct ligand for HER2 has not been identified; and HER2-containing heterodimers are formed preferentially. Different receptor combinations have different activities in cell cultures *in vitro*: homodimers are less mitogenic than heterodimeric combinations and have a low transforming activity, with HER2-containing heterodimers being most potent

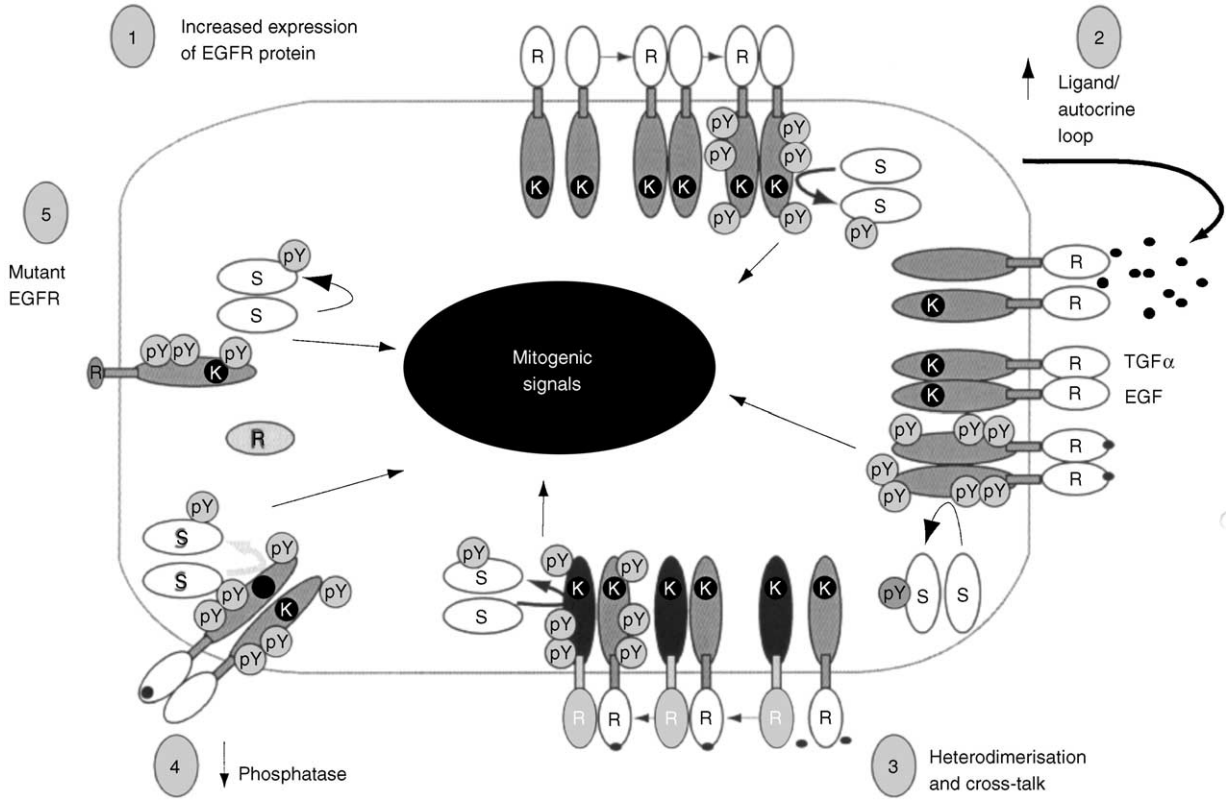

Fig. 2. Potential mechanisms of increased epidermal growth factor receptor (EGFR) activation. TGFα, transforming growth factor α; K, kinase; R, receptor; S, substrate.

[8]. Therefore, the levels of other erbB family receptors, particularly HER2, may significantly affect EGFR signalling in human cancers.

In addition to activation by its own ligands, the EGFR signalling network might also be influenced by other cellular signalling systems, one of the most studied being G-protein-coupled receptors (GPCRs) [8,28,29]. GPCRs can have positive effects on EGFR signalling and mechanisms may involve activation of matrix metalloproteinases, which are then able to cleave membrane-tethered ligands, freeing them to bind erbB receptors, or indirect activation of Src, which phosphorylates the intracellular receptor tyrosine residues. Transactivation of EGFR signalling can also occur through steroid hormones, which can activate the transcription of genes encoding EGFR ligands, such as TGFα [30].

5. EGFR-targeted agents: the relationship between activity and EGFR expression

EGFR-targeted agents have been the focus of intensive research and the orally active EGFR tyrosine kinase inhibitor (EGFR-TKI) gefitinib ('Iressa', ZD1839) is the first of such agents to be approved; for the treatment of inoperable/recurrent non-small cell lung cancer in Japan. A relevant clinical question is to determine the role of EGFR expression on the activity of such agents. On the basis of the observations above, it is unclear whether EGFR expression *per se* will predict for response.

Preclinical studies with gefitinib demonstrated the often complex relationship between the expression of EGFR and the activity of an EGFR-targeted agent and have suggested that the efficacy of gefitinib is not directly related to EGFR expression levels. High EGFR expression is no guarantee of sensitivity to gefitinib, since defects downstream of the receptor may affect signal transduction and other pathways may compensate to drive tumour proliferation. Activity of gefitinib has been observed in cells that express high and low levels of EGFR [31] and, conversely, lack of effect has been observed in cell lines with varying degrees of EGFR expression [32]. Studies using cell lines derived from a variety of different carcinomas have in some cases demonstrated a relationship between relative EGFR expression and activity of gefitinib [33], whereas others have reported no such effect [34,35]. Meye and colleagues [33] investigated the effects of gefitinib in four bladder cancer cell lines, each expressing a different level of EGFR. The IC_{50} for ligand-independent growth ranged from 1.8 to 9.7 μM in these cell lines and correlated with EGFR protein and transcript level. Despite

different levels of EGFR expression in two cell lines derived from head and neck squamous-cell carcinoma and melanoma, the levels of growth inhibition were similar [35]. Interestingly, in two preclinical studies in which gefitinib caused growth inhibition of human tumour xenografts and markedly enhanced the activity of a number of cytotoxic agents, neither the growth inhibition nor the degree of potentiation was dependent upon a high level of EGFR expression [34,36]. These results suggest that EGFR-targeted drugs could be effective in patients with tumours that express relatively low levels of EGFR.

Several researchers have shown that breast tumour cell lines that express high levels of HER2 are particularly sensitive to gefitinib, an effect thought to be mediated by the binding of gefitinib to the EGFR component of the EGFR/HER2 dimer [31,32,37,38]. Further support for the involvement of EGFR in the transactivation of HER2 was provided by Normanno and colleagues [39]. Gefitinib treatment of SK-Br-3 cells, which express both EGFR and HER2, produced a significant, dose-dependent reduction of the tyrosine phosphorylation of both receptors (Fig. 3a). In contrast, NIH/3T3 cells that expressed HER2, but not EGFR, did not undergo a reduction in HER2 phosphorylation when treated with gefitinib (Fig. 3b). These data suggest that the activity of agents like gefitinib should be tested in patients with tumours that are known to express high levels of HER2 and are likely to express EGFR.

Clinical trials of gefitinib were carried out with patients who were not selected on the basis of EGFR expression [11,40,41], in contrast to trials of the small-molecule EGFR-TKI OSI-774 and the monoclonal antibody C225, some of which have been carried out in patients selected as EGFR-positive [42,43]. Interestingly, the phase II trial of OSI-774 did not find an association between objective tumour response or stable disease and the level of EGFR staining [42]. In contrast, a phase II study of C225 found that responders (two partial responses, one complete response) had higher expression levels of EGFR mRNA than non-responders [44].

5.1. Potential markers for response to EGFR-targeted agents and development of resistance to EGFR-targeted agents

To date, no clear association between EGFR levels and response to EGFR-targeted agents has been described. In predicting the response to EGFR-targeted agents, it is likely that the levels of activated, phosphorylated EGFR are more important than total EGFR levels. Rojo and colleagues have suggested that pharmacodynamic endpoints may correlate with antitumour activity, supported by the observation that, in a human breast cancer xenograft model, doses of gefitinib that resulted in the inhibition of activated EGFR, phosphorylated MAPK and Ki67 correlated with maximum tumour growth inhibition [45]. Moreover, the integrity of the EGFR-activated downstream intracellular signal transduction machinery could influence the response to these drugs. In this respect, recent experimental evidence suggest that the cancer cell may escape from growth inhibition by using alternative growth pathways or by constitutive activation of downstream signalling effectors. As an example, human A431 cancer

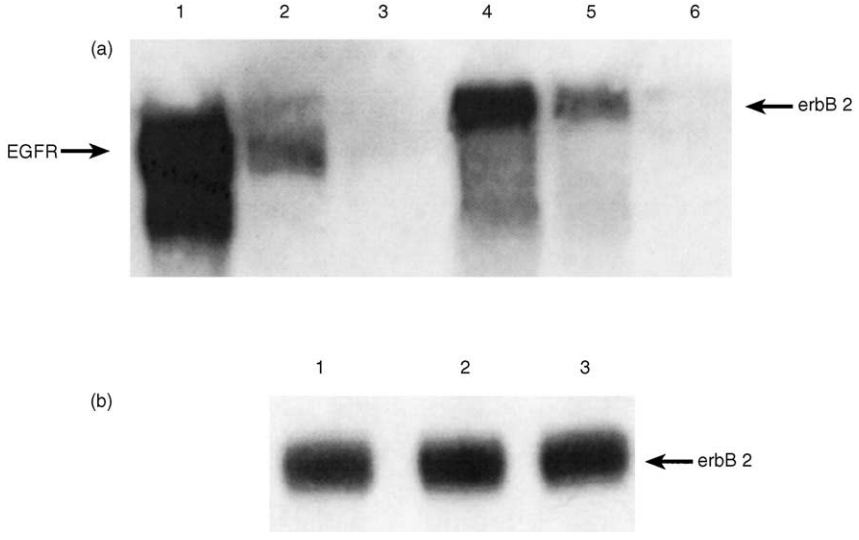

Fig. 3. (a) Effects of gefitinib on tyrosine phosphorylation of epidermal growth factor receptor (EGFR) and HER2 in SK-Br-3 cells. EGFR (lanes 1–3) or HER2 (lanes 4–6) was immunoprecipitated with specific antibodies and western blot analysis was performed with a specific antiphosphotyrosine antibody. Lanes 1 and 4, untreated cells; lanes 2 and 5, cells treated with 1 μM gefitinib; lanes 3 and 6, cells treated with 5 μM gefitinib. (b) Effects of gefitinib on tyrosine phosphorylation of HER2 in HER2-transfected NIH/3T3 cells. Lane 1, untreated cells; lane 2, cells treated with 1 μM gefitinib; lane 3 cells treated with 5 μM gefitinib [39]. Reproduced with permission from Oxford University Press.

cells xenografts can acquire resistance to anti-EGFR MAbs such as C225 and hR3 by increased tumour-induced angiogenesis due to the constitutive over-expression of pro-angiogenic growth factors vascular endothelial growth factor (VEGF) by cancer cells [46]. It has been also proposed that activation of the type I insulin-like growth factor receptor may determine continuous activation of the anti-apoptotic phosphoinositide 3-kinase/AKT signalling pathway that efficiently counteracts the antiproliferative effects of EGFR inhibitors in human glioblastoma cells *in vitro* [47]. This could have significant clinical implications. Since it is conceivable that in cancer cells multiple growth controlling pathways are altered, the combination of biological therapeutics targeting two or more of such pathways should be tested in a clinical setting in order to develop a poly-targeted therapy that is based on a rational approach to the alterations that are present in a cancer patient. In this context, in preclinical experimental models, it has been demonstrated that a significant and sustained anti tumour activity *in vitro* and *in vi*vo can be obtained by the combination of anti-EGFR agents with other anti signalling agents, such as inhibitors of the cAMP-dependent protein kinase (type I PKA) [48,49], a VEGF antisense oligonucleotide [50] or the anti-ErbB-2 MAb trastuzumab [37,39].

6. Conclusions

There is significant evidence for EGFR as a valid target in cancer therapy from the early clinical trials of EGFR-targeted agents. However, much remains to be learned about the relevance of EGFR as a prognostic factor or as a predictive marker for the sensitivity/response to EGFR-targeted agents. After the first series of phase I, II and III clinical trials, the next generation of clinical studies with anti-EGFR drugs in cancer patients should be designed with a strong translational research effort to address several key clinical questions, such as which patients are most likely to have a therapeutic benefit, what are the potential predictive factors of response or resistance that could be useful in a clinical setting and what are the best strategies for combining anti-EGFR drugs with conventional anticancer treatments.

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