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Review

Resistance to EGF-R (erbB-1) and VEGF-R modulating agents

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ABSTRACT

In an effort to improve the survival of cancer patients, new therapeutic approaches focusing on the molecular mechanisms that mediate tumour cell growth or survival have gained much attention. In particular, EGF-R and VEGF/VEGF-R have been extensively investigated as targets for anti-neoplastic therapy. Agents that selectively target EGF-R, erbB-2, VEGF-R-2 or VEGF have shown promising activity in clinical trials, and several are now approved for use in selected cancer indications. However, all patients ultimately develop resistance to these drugs. Thus, there is a great need to understand how patients become resistant to effective therapies for these cancers since this approach may lead to improvements in therapies that target EGF-R and VEGF/VEGF-R. Pre-clinical studies have begun to shed light on the mechanisms of resistance to anti-angiogenetic drugs and to date four mechanisms of resistance have been identified (1) upregulation of bFGF, (2) overexpression of MMP-9, (3) increased levels of SDF-1α and (4) HIF-1α-induced recruitment of bone marrow-derived CD45+ myeloid cells. In addition, the molecular mechanisms of resistance to EGF-R modulating agents can be attributed to several general processes: (1) activation of alternative tyrosine kinase inhibitors that bypass the EGF-R pathway (e.g. c-MET and IGF-1R), (2) increased angiogenesis, (3) constitutive activation of downstream mediators (e.g. PTEN and K-ras) and (4) the existence of specific EGF-R mutations. K-ras mutations have been significantly associated with a lack of response to EGF-R tyrosine kinase inhibitors in patients with NSCLC and with a lack of response to cetuximab or to panitumumab in patients with advanced colorectal cancer. The identification of these resistance mechanisms has led to clinical trials using newly designed targeted therapies that can overcome resistance and have shown promise in laboratory studies. Ongoing research efforts will likely continue to identify additional resistance mechanisms, and these findings will hopefully translate into effective therapies for different cancers.

1. Introduction

Despite advances in chemotherapy, most patients with cancer that has metastasised will succumb to the disease within 2 years of diagnosis. In an effort to improve survival, new therapeutic approaches focusing on the molecular mechanisms that mediate tumour cell growth or survival have gained much attention. In particular, the epidermal growth factor receptor (EGF-R) and the vascular endothelium growth factor receptor (VEGF-R) have been extensively investigated as targets for anti-neoplastic therapy.

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Receptor tyrosine kinases (RTKs) such as EGF-R or VEGF-R are transmembrane proteins with an extracellular ligand binding domain and an intracellular tyrosine kinase catalytic domain. On binding to their cognate ligands, most RTKs dimerise and become activated through autophosphorylation of intracellular tyrosine residues. Activation of RTKs results in upregulation of multiple cellular signalling pathways that promote cell growth, survival and angiogenesis or environmental stimuli. Inappropriate activation of RTKs via mutation, overexpression or ectopic ligand production is a frequent feature of human tumour development and progression, and is thought to be a major mechanism by which cancer cells subvert normal growth controls.[1–3] Consequently, in recent years modulation of RTK signal transduction has been an active area in oncology drug discovery. EGF-R (also called erbB1) and other erbB family RTKs (erbB-2/HER-2-neu, erbB-3/HER-3 and erbB-4/HER-4) encoded by the c-erbB proto-oncogenes have been strongly implicated in cancer development and progression as reviewed by [4,2]. Several mechanisms can cause aberrant receptor activation, resulting in tyrosine kinase activity, which is observed in cancer, including receptor overexpression, mutation, ligand-dependent receptor dimerisation and ligand-independent activation. For erbB-2, where a specific ligand has not been identified, activation occurs by homo- or hetero-dimerisation alone, whereas erbB-3 does not have significant kinase activity.[4,5] However, on activation, all 4 receptors are capable of signal transduction, causing activation of the ras/MAP kinase pathway, the PI3K/Akt pathway, src family kinases and STAT proteins. Activation of these pathways promotes cell proliferation, survival and angiogenesis.[6]

VEGF is the prototype of a large family of angiogenic and lymphangiogenic growth factors, which includes 6 structurally homologous, secreted glycoproteins called VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor.[1] VEGF-A (commonly referred to as VEGF) was the first such molecule to be identified by the virtue of its ability to induce vascular permeability.[7] The VEGF ligands trigger biological effects on their interaction with specific cell-surface receptors. The diversity of these receptors also adds to the biological complexity of angiogenesis and lymphangiogenesis. Two receptors were originally identified on vascular endothelial cells: VEGF-R-1 (a 180-kD transmembrane protein, also called Flt-1) and VEGF-R-2 (a 200-kD transmembrane protein, also called KDR). A third structurally related tyrosine kinase receptor is the 180-kD VEGF-R-3 (also called Flt-4), which is expressed broadly on endothelial cells during early embryogenesis.[8] VEGF-R-2 is expressed in most, if not all, adult vascular endothelial cells as well as on circulating endothelial progenitor cells. Interestingly, both epithelial and mesenchymal tumour cells more typically express VEGF-R-1 than VEGF-R-2[9]; however, in several experimental tumour models tumour cell-specific VEGF-R-2 expression has been shown to be the critical driver in the pathogenesis of tumours.[1,3] VEGF binding induces conformational changes within VEGF-R-2 followed by receptor dimerisation and autophosphorylation of tyrosine residues in the intracellular kinase domain. These tyrosine residues (Tyr^{951}, Tyr^{996}, Tyr^{1054} and Tyr^{1059}) serve as high-affinity docking sites for a variety of signalling proteins, including phospholipase Cγ, ras-GAP, focal adhesion kinase, src family of tyrosine kinases, PI3K. Akt, PK-C, Raf-1 and MAPs. The interaction of 1 or more of these molecules with VEGF-R-2 may lead to alterations in cell proliferation, migration, differentiation, tube formation, and increase in vascular permeability and vascular integrity.[3]

Agents that selectively target EGF-R, erbB-2, VEGF-R-2 or VEGF have shown promising activity in clinical trials, and several are now approved for use in selected cancer indications (Tables 1 and 2). All patients, however, ultimately develop resistance to anti-EGF-R-and anti-VEGF(-R)-targeted therapies. Thus, there is a great need to understand how patients become resistant to effective therapies for these cancers.

2. Resistance to VEGF-R modulating drugs

Most current antiangiogenic strategies for cancer therapy are based on blocking VEGF functions, and anti-VEGF agents have successfully been used for the treatment of certain types of human cancers (Fig. 1). However, tumours also produce multiple non-VEGF angiogenic factors, and anti-VEGF monotherapy could potentially encounter drug resistance, suggesting that tumours could use non-VEGF angiogenic factors to grow blood vessels.

Intrinsic and acquired resistance to anti-angiogenetic drugs are clinically significant problems. Pre-clinical studies have begun to shed light on the mechanisms of such resistance, and to date 4 mechanisms of resistance have been identified (a) upregulation of the basic fibroblast growth factor (bFGF), (b) overexpression of matrix metalloproteinase-9 (MMP-9), (c) increased levels of SDF-1α (stromal-cell-derived factor) and (d) hypoxia-induced factor (HIF)-1α-induced recruitment of bone marrow-derived CD45+ myeloid cells.

Inhibition of VEGF-R-2 (but not VEGF-R-1) markedly disrupts angiogenic switching, persistent angiogenesis and initial tumour growth. In late-stage tumours, phenotypic resistance to VEGF-R-2 blockade emerges as tumours regrow during treatment after an initial period of growth suppression. This resistance to VEGF blockade involves reactivation of tumour angiogenesis, independent of VEGF, and is associated with hypoxia-mediated induction of other proangiogenic factors, including members of the FGF family. These other proangiogenic signals are functionally implicated in the revascularisation and regrowth in the evasion phase, as FGF blockade impairs progression in the face of VEGF-inhibition.

The FGF family comprises 23 distinct, structurally related proteins described to date that exert biologic effects in different cells and organ systems, including tumour growth and angiogenesis.[10] FGFs are heparin-binding proteins, which interact with low-affinity heparan sulphate proteoglycans (HSPGs). HSPGs are ubiquitous cell-surface and extracellular matrix (ECM) proteins, which have been shown to protect FGFs from thermal denaturation and proteolysis, as well as to increase FGF receptor affinity and facilitate FGF-binding to cell-surface receptor. In addition, ECM-associated HSPGs modulate FGF bioavailability by generating a local reservoir for the growth factor and by allowing a sustained stimulation of endothelial cells.[11] Mobilisation of FGFs from the ECM storage, and in particular of FGF-1 and FGF-2, occurs via HSPG digestion by heparanases or glycosaminoglycan-degrading

Table 1 – EGF-R inhibitors currently approved for cancer treatment.

Drug	Category (target)	Status
Erlotinib (Tarceva®)	Tyrosine kinase inhibitor (EGF-R, erbB-1)	Approved for NSCLC, pancreatic cancer
Gefitinib (Iressa®)	Tyrosine kinase inhibitor (EGF-R, erbB-1)	Approved for NSCLC (Asian countries)
Lapatinib (Tyverb® and Tykerb®)	Tyrosine kinase inhibitor (erbB-1, erbB-2)	Approved for MBC (preliminary approvable)
Cetuximab (Erbitux®)	Human-mouse chimeric monoclonal antibody (IgG1 subtype) (EGF-R)	Approved for CRC (K-ras wild-type patients only), head and neck tumours
Panitumumab (Vectibix®)	Fully human monoclonal antibody (IgG2κ subtype) (EGF-R)	Approved for CRC (K-ras wild-type patients only)

Table 2 – VEGF-R inhibitors and anti-VEGF agents currently approved for cancer treatment.

Drug	Category (targets)	Status
Sorafenib (Nexavar®)	Tyrosine kinase inhibitor (VEGF-R-2, raf, c-KIT, PDGF-Rβ)	Approved for RCC and HCC
Sunitinib (Sutent®)	Tyrosine kinase inhibitor (VEGF-R-2, PDGF-R, c-KIT, RET, Flt-3)	Approved for GIST and RCC
Bevacizumab (Avastin®)	Monoclonal antibody (VEGF)	Approved for CRC, NSCLC and MBC

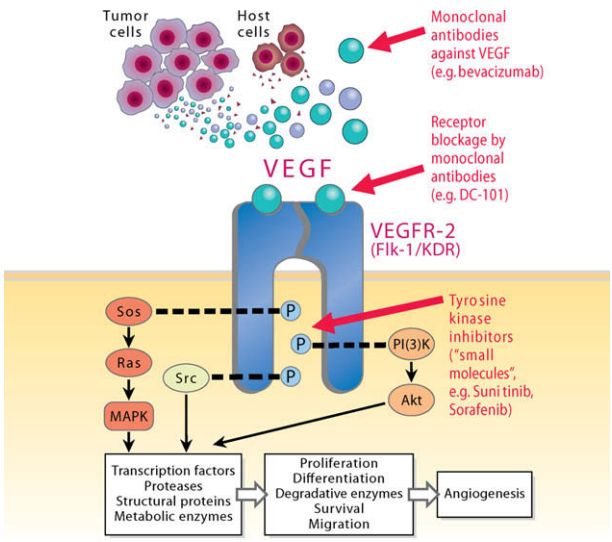

Fig. 1 – Signalling pathways activated by VEGF.

enzymes. FGFs act through high-affinity binding sites that mediate biological activity via a group of tyrosine kinase membrane receptors that form the FGF-R family. Within the FGF-R family, 4 members have been identified: FGF-R-1, FGF-R-2, FGF-R-3 and FGF-R-4. Structural features shared by the FGF-R family include 3 glycosylated immunoglobulin-like loops of the extracellular domain and an internal conserved tyrosine kinase domain split by a short insert.[12] It has been shown that the 4 members of the FGF-R family bind both FGF-1 and FGF-4. FGF-2 is able to bind FGF-R-1, FGF-R-2 and FGF-R-3, whereas FGF-5, FGF-6 and FGF-7 act through FGF-R-3, FGF-R-4 and FGF-R-2, respectively.[11]

Transcriptional regulation of VEGF is critically dependent on HIF-1. However, not only hypoxia, but also selected growth factors can induce HIF-1.[13] Results from several studies have provided compelling evidence that hypoxia-triggered upregulation of other proangiogenic factors (e.g. FGF family and PDGF-BB) in the presence of anti-VEGF agents can restimulate tumour angiogenesis in a VEGF-independent fashion and thereby contribute to the resistance to VEGF-blocking agents[14–16] (Fig. 2).

In terms of the underlying molecular mechanisms it has been demonstrated that hypoxia induces the expression of HIF-1α (a key protein for tumour angiogenesis), and the release of bFGF further augments these hypoxic inductions. The PI3K pathway has been shown to be required for these processes as demonstrated by the application of the PI3K inhibitor LY294002.[16] In addition, under hypoxic conditions, bFGF activates the MEK1/ERK pathways, and PD98059 (a MEK1,2 inhibitor) suppresses the bFGF-induced HIF-1 transactivity, suggesting that the ras signalling cascade may also be involved in the resistance to anti-VEGF agents.

Furthermore, it has been shown in experimental systems that adding a bFGF inhibitor (brivanib, BMS-582664) to tumours expressing resistance to bevacizumab, SU6668 and ZD6474 can significantly restart re-initiation of angiogenesis and tumour progression[14] (Dr. Mark Ayers, unpublished work). Currently, only very few drugs are available to target the FGF receptor (CHIR258, PD173074, BIBF-1120 and BMS-582664). Amongst them brivanib (BMS-582664) is a novel orally available and selective tyrosine kinase inhibitor that targets the key angiogenesis receptors VEGF-R-2 and FGF-R-2. The drug is currently under clinical evaluation (phase III) for different cancers (colorectal carcinoma and hepatocellular cancer), and has shown promising clinical activity and manageable side-effects.[17] Since resistance to VEGF blockade involves vascular regrowth in a VEGF-independent second wave of angiogenesis (mediated in part by proangiogenic ligands of the FGF family), counteracting such mechanisms by multitargeting alternative proangiogenic signalling circuits may improve the efficacy of antiangiogenic therapies.

The matrix metalloproteinases (MMPs) comprise a relatively large and ever-growing family. There are now more than 20 enzymes that are classified as MMPs. These enzymes

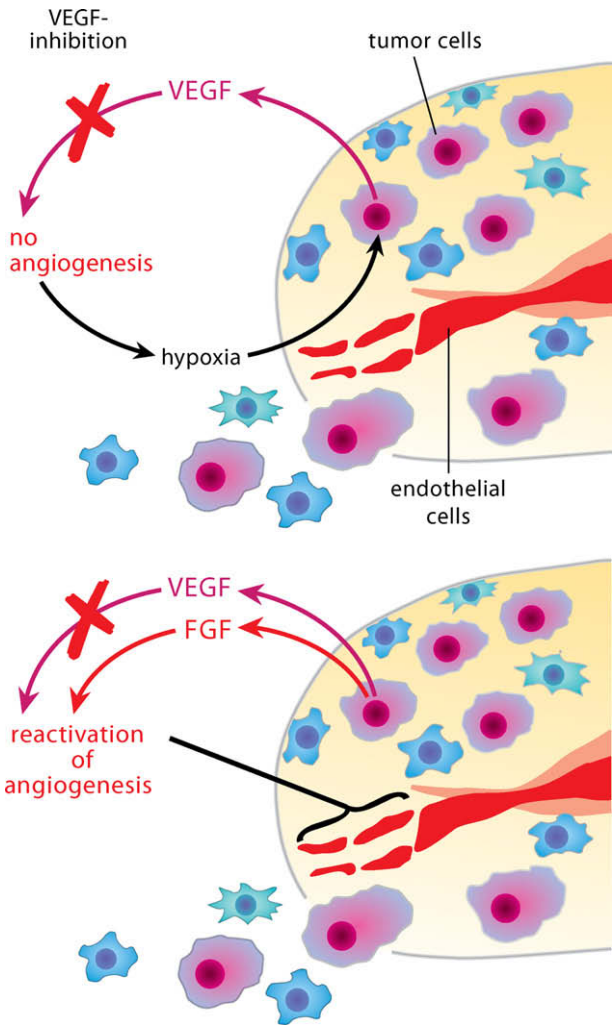

Fig. 2 – Suppression of angiogenesis by anti-VEGF agents. Overexpression of FGF can restore angiogenesis and thereby confer resistance to VEGF/VEGF-R inhibitors.

have both a descriptive name and a MMP number. Although the numbering system recognises up to MMP-24, the nomenclature does not accurately reflect the actual number of enzymes, because MMP-4, MMP-5 and MMP-6 have been eliminated as a result of duplication. All MMPs have a similar domain structure, with a 'pre' region to target secretion, a 'pro' region to maintain latency and an active catalytic region that contains the zinc-binding active site. The majority of MMPs have additional domains, such as a haemopexin region or a fibronectin-like region. These additional domains are important in substrate recognition and in inhibitor binding as reviewed by [18]. MMPs regulate angiogenesis: on the one hand, by facilitating extracellular matrix degradation to allow new vessel expansion,[19] and on the other hand, by interfering with angiogenesis through the production of angiostatin. Angiostatin is generated by the proteolytic cleavage of plasminogen by several members of the MMP family including MMP-2 and MMP-9.[20] In the vasculature, MMP-2 and MMP-9 are produced by smooth muscle and endothelial cells.[21] Under hypoxic conditions gene expression of MMP-9 is upregulated (stimulated by VEGF) and is mediated by autocrine VEGF signalling. In contrast, exogenous MMP-9 increases the gene expression and secretion of VEGF.[22] MMP-9 activity of bone marrow-derived CD45+ cells is essential and sufficient to initiate angiogenesis by increasing VEGF bioavailability. In the absence of HIF-1α, SDF-1α levels decrease, and fewer bone marrow-derived cells are recruited to the tumours, decreasing MMP-9 and mobilisation of VEGF.[23,24]

Since the hypoxic expression of MMP-9 can stimulate the production and secretion of VEGF in tumours, overexpression of MMP-9 may contribute to the resistance to anti-VEGF agents. Recently, it has been shown that inhibitors of MMP-9 (e.g. minocycline or pyrrolidine dithiocarbamate) can down-regulate VEGF levels in human tumours and non-malignant tissues,[25] suggesting that MMP-9 could be an important target for adjunct therapy to enhance the response of tumours to anti-VEGF agents.

Chemokines are a family of small peptides, many of them were first identified as chemoattractants for leucocytes but are now recognised to have a number of diverse functions. They are divided into 4 groups, CXC, CX_3C, CC and C (C = cysteine and X = any amino acid), based on the positioning of 2 highly conserved cysteines near the amino terminus. Chemokines are ligands for a family of 7 transmembrane-spanning G-protein-coupled receptors. Numerous chemokine receptors have been identified including 7 for CXC chemokines, eleven for CC chemokines and one each for CX_3C and C.[26,27] One of these chemokines acts as receptor (CXCR4) for the SDF-1. Both SDF-1 and CXCR4 have hypoxia response elements in the promoter regions of their genes and are increased under hypoxic conditions.[28,29] It has been shown that increased expression of VEGF in tumours results in increased levels of SDF-1 and CXCR4, and the increase in CXCR4 is due primarily to the recruitment of bone marrow-derived cells that express CXCR4. The recruitment appears to be due to VEGF signalling through VEGF-R-1,[30] and the increase in SDF-1 that occurs in pericytes and smooth muscle cells functions to localise and retain bone marrow-derived cells adjacent to blood vessels, where they provide proangiogenic factors that work with VEGF to stimulate neo-vascularisation.[28,31] SDF-1 and CXCR4 mRNA expression is upregulated by VEGF and thereby contributes to cell invasion. In contrast, the CXCR4 antagonist AMD3100 could inhibit cell invasion, but not proliferation.[32] It has been suggested that SDF-1-mediated vasculogenesis may represent an alternate pathway that could potentially be utilised by tumours to sustain growth and neovasculature expansion after anti-VEGF therapy and therefore, contribute to resistance to anti-VEGF agents. Clinical trials are clearly needed to further determine whether combined blockade of CXCR4 and VEGF provides additional benefit.

New blood vessel formation in tumours and ischemic tissues is derived from the existing vasculature by activating proliferation and migration of endothelial cells (angiogenesis) and by recruiting a heterogenous population of bone marrow-derived cells (BMDCs), including endothelial progenitor cells (EPCs), pericyte progenitor cells (PPCs) and CD45+ vascular modulatory cells[33,34,24] (Fig. 3). While EPCs incorporate into the vasculature and differentiate into endothelial cells, PPCs envelop blood vessels and mature into pericytes and vascular smooth muscle cells. CD45+ cells of monocytic lineage make up the largest and most heterogeneous group of BMDCs that

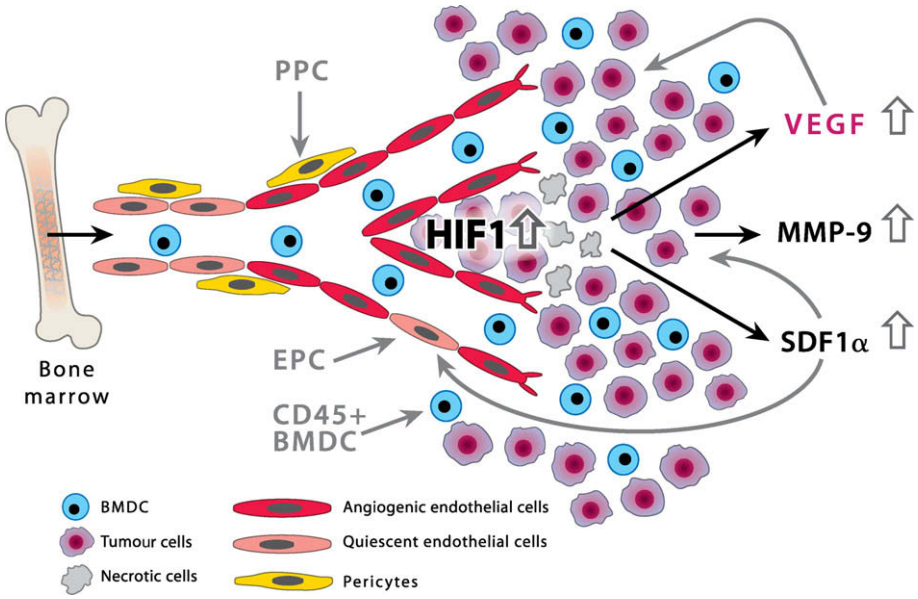

Fig. 3 – HIF-1 is a critical regulator of BMDC recruitment in tumours.

function as vascular modulators, but are not physically part of the vasculature.[33] Such cells include tumour-associated macrophages, immature monocytic cells including Tie2+ haemangiocytes and CD11b+ myeloid cells, all of which repress the SDF-1-receptor CXCR4 to some extent.[24] Little is known about the factors that enable the mobilisation of BMDCs from the bone marrow into the blood stream to their recruitment and retention into the tumour. The most prominent factors identified so far include the HIF-1/VEGF complex, angiopoietin-1, PIGF and PDGF-B.[35,13] HIF-1 is a transcription factor that regulates oxygen homeostasis in response to changes to oxygen levels in normal and tumour tissues.[36] HIF-1 is a ubiquitously expressed and highly conserved heterodimeric basic-helix-loop-helix-PAS transcription factor composed of an α- and a β-subunit. As cellular oxygen concentration decreases, levels of the HIF-1α subunit increase, and this determines the level of HIF-1 activity.[36,37] Under hypoxic conditions, HIF-1 activates a large battery of genes whose protein products function either to increase O_2 availability or to allow metabolic adaptation of cells to oxygen deprivation within their microenvironment. These genes contain certain hypoxic response elements (HREs) and include genes such as VEGF and $CXCR_4$/SDF-1.[38,39] As a result, HIF-1α and its signalling pathway have become targets for cancer chemotherapy aimed at inhibiting angiogenesis. In a most recently published study by Bergers and co-workers,[24] it has been demonstrated that HIF-1 (in part by inducing SDF-1α) is a major recruitment regulator of bone marrow-derived EPCs, PPCs and monocytic vascular modulatory cells to endorse vascular remodelling in tumours. HIF-1 not only induced VEGF transcription in these tumours, but also increased VEGF activity by recruiting CD45+ BMDCs that carried and secreted the MMP-9 to the tumour site, which in turn made sequestered VEGF bioavailable for its receptor VEGF-R-2. Furthermore, they found that MMP-9 was expressed in all CD45+ monocytic cell types that have been implicated in angiogenesis (Fig. 3). Increased bioavailability of VEGF due to influx of MMP-9-expressing CD45+ cells not only induced angiogenesis, but also regulated tumour cell invasiveness. VEGF prevented tumour cell migration along blood vessels, but appeared to promote tumour cell infiltration into the parenchyma. Therefore, tumour cells use perivascular invasiveness as an evasive adaptation mechanism when angiogenesis is impaired. Using a HCT116 xenograft model, Dang and colleagues[40] have provided evidence that the extent of tumour vessel response to angiogenetic inhibition could be correlated with (a) the pre-existing coverage of tumour endothelial tubes with pericytes and (b) differential induction of HIF-1 target genes, suggesting that hypoxia is a driving force in BMDC-dependent neovascularisation of tumours.

Following prolonged treatment with RTKs (sunitinib, sorafenib, axitinib and vatalanib), typically secondary mutations in the tyrosine kinase-binding domain occur and thereby confer resistance to these drugs as described for GISTs and renal cell carcinomas, for reviews see [41–44]. However, a triad of molecular changes involving elevated levels of SDF-1α, HIF-1, VEGF or proangiogenetic BMDCs have also been documented,[45] suggesting that the aforementioned resistance mechanisms not only are associated with resistance to VEGF antibodies but may also be involved in the resistance to small molecule-RTKs used clinically and may contribute to the observed rapid tumour (re)growth seen after cessation of therapy.

3. Resistance to EGF-R (erbB-1) modulating drugs

Anti-EGF-R-targeted therapies have improved the efficacy of conventional chemotherapy in both pre-clinical and clinical studies. Although such therapies may lead to partial response or disease stabilisation in some patients, many patients do not benefit from anti-EGF-R therapy, to those who do eventually

develop resistance to that therapy. Great interest therefore, exists in elucidating resistance mechanisms for anti-EGF-R therapies as well as those for chemotherapy agents. The molecular mechanisms of resistance can be attributed to several general processes: (a) resistance due to the activation of alternative tyrosine kinase receptors that bypass the EGF-R pathway (e.g. c-Met and IGF-1R) (b) resistance due to increased angiogenesis, (c) resistance based on constitutive activation of downstream mediators (e.g. PTEN, K-ras and others) and (d) the existence of specific EGF-R mutations. Interestingly, most of these resistance mechanisms (e.g. IGF-1R overexpression, PTEN loss, bypassing of EGF-R pathways, receptor masking or epitope inaccessibility) are also implicated in the trastuzumab resistance and are reviewed elsewhere.[46] Understanding the molecular mechanisms of resistance and sensitivity may lead to improvements in therapies that target EGF-R.

A substantial body of evidence suggests that the amplification of the Met oncogene could lead to EGF-R inhibitor resistance by activating erbB-3 signalling.[47] Met encodes a heterodimeric transmembrane receptor tyrosine kinase composed of an extracellular α-chain disulphide bonded to a membrane-spanning β-chain.[48] Binding of the receptor to its ligand, hepatocyte growth factor/scatter factor, induces receptor dimerisation, triggering conformational changes that activate Met tyrosine kinase activity. Met tyrosine kinase activity can have profound effects on cell growth, survival, motility, invasion and angiogenesis[49] (Fig. 4). Dysregulation of Met signalling has been shown to contribute to tumourigenesis in a number of malignancies. Met amplification leads to EGF-R-independent activation of the PI3K/Akt pathway through the activation of erbB-3-dependent signalling. Recently, Wheeler et al.[50] have established resistant tumour cells following chronic exposure to cetuximab. Cells developing acquired resistance to cetuximab exhibited increased steady-state EGF-R expression secondary to alterations in trafficking and degradation. In addition, cetuximab-resistant cells manifested strong activation of erbB-2, erbB-3 and c-Met. EGF-R upregulation promoted increased dimerisation with erbB-2 and erbB-3, leading to their transactivation.

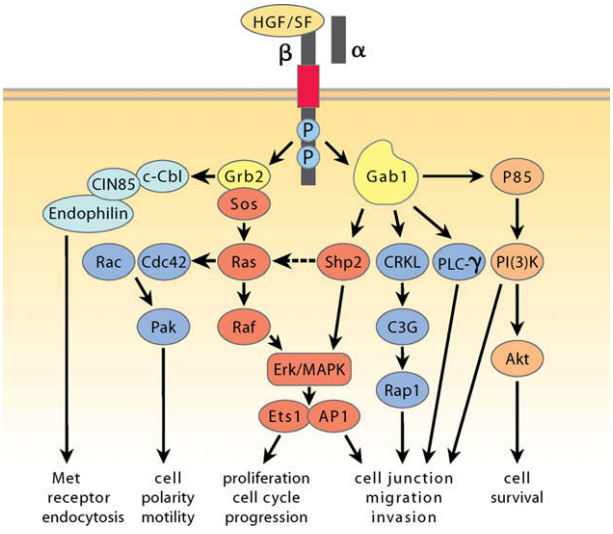

Fig. 4 – Met-modulated signal transduction pathways.

These data suggest that acquired resistance to cetuximab (and probably to panitumumab) is accompanied by dysregulation of EGF-R internalisation/degradation and subsequent EGF-R-dependent activation of erbB-3 (crosstalk with the c-Met pathway). In addition, preliminary results from the ongoing studies suggest that cetuximab is less active in patients harbouring a mutated EGF receptor,[51] (Dr. Jeff Engelman, Boston, unpublished work).

Loss of IGF-binding proteins (IGFBPs) was reported to be involved in the resistance to EGF-R-targeted tyrosine kinase inhibitors.[52] The authors isolated gefitinib-resistant human squamous carcinoma A431 cells by prolonged incubation with an increasing amount of the inhibitor. In the gefinitib-resistant cells, the inhibitor reduced the phosphorylation levels of EGF-R, erbB-3 and Erk, but not those of Akt. This adaptive change was accompanied by activation of the signalling events mediated by the IGF-1 receptor (IGF-1R), such as phosphorylation of IRS-1 and the interaction of IRS-1 with PI3K. In addition, Guix and colleagues found that the expression levels of IGF-binding protein 3 (IGFBP-3) and -4, 2 of the negative regulators of the IGF-1R signalling, were reduced in the gefitinib-resistant cells. Furthermore, it was shown that inhibition of IGF-1R disrupted the association of IRS-1 with PI3K and restored the ability of gefitinib to reduce Akt phosphorylation and to inhibit cell growth (Fig. 5). Interestingly, the gefinitib-resistant cells were cross-resistant to erlotinib and the monoclonal antibody cetuximab, suggesting that the loss of IGFBPs is involved in the resistance to other erbB-targeting tyrosine kinase inhibitors and antibodies. These findings are consistent with earlier reports that activation of the PI3K pathway, which has been shown to be dominant in transformation-related signalling events caused by erbB kinase complexes, is a critical mediator of resistance to EGF-R modulating agents.[53]

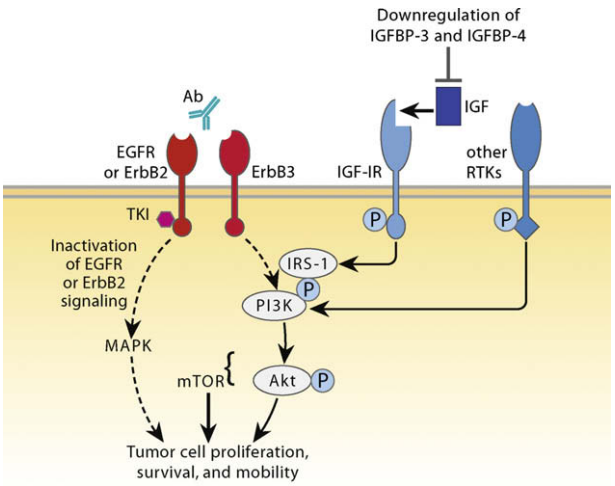

Fig. 5 – Mechanisms of erbB-targeted therapy. ErbB-targeted drugs cause downregulation of the MAPK, mTOR and PI3K signalling pathways (dashed lines indicate reduction in signalling). Resistance may arise in tumour cell through allelic and adaptive changes, leading to activation of PI3K through other receptor tyrosine kinases. Downregulation of IGFBP-3 and -4, negative regulators of IGF-1R signalling, causes activation of IGF-1R and the PI3K pathway and contributes to the resistance to anti-EGF-R agents.

It would therefore, be of interest to investigate how prevalent this mechanism of acquisition of resistance to gefitinib is in physiologic conditions.

An increasing body of evidence suggests that EGF-R-mediated pathways are intimately involved in tumour angiogenesis through upregulation of VEGF and other mediators of angiogenesis.[54] Treatment of a variety of EGF-R-expressing tumour cells with cetuximab resulted in downregulation of various angiogenic mediators, and the efficacy of cetuximab is more pronounced in xenografts than in cell culture, an effect that could be explained, at least in part, by the antiangiogenic consequences of the EGF-R blockage. Since upregulation of tumour angiogenesis-promoting growth factors is a potential mechanism by which tumour cells may overcome the deleterious effects of EGF-R inhibition,[55] combining molecular therapies targeting several survival pathways, such as anti-VEGF monoclonal antibodies or VEGF-R-2 inhibitors, with EGF-R inhibitors may result in potential benefit for cancer patients. In an approach to test this hypothesis, cetuximab plus DC-101 (anti-VEGF-R-2 antibody) was used to treat gastric cancer grown in nude mice.[56] Both antibodies were modestly effective in inhibiting tumour growth, but the combination achieved significantly greater tumour growth inhibition that was also associated with decreased tumour vascularity and increased tumour cell apoptosis. In contrast to these xenograft results, Punt et al.[57] presented the CAIRO-2 data at this year's ASCO meeting. In this randomised phase III study, 755 patients with metastatic colon cancers were treated with capecitabine, oxaliplatin (CapOx) and bevacizumab or with CapOx, bevacizumab plus cetuximab. The combination of both antibodies, cetuximab and bevacizumab, to CapOx resulted in a significant decrease in PFS compared to bevacizumab and CapOx, however, overall survival rates did not differ, suggesting that cetuximab may not enhance antitumour effects of anti-VEGF blockade.

PTEN is a lipid phosphatase and tumour suppressor protein that regulates the PI3K/Akt signalling pathway.[58] The major substrate for PTEN is phosphatidylinositol 3,4,5-triphosphate, a second messenger of PI3K. With the loss of PTEN function, phosphatidylinositol 3,4,5-triphosphate accumulates in the cell membrane, when it binds and activates Akt. Thus, loss of PTEN function results in overactivation of the Akt pathway, increasing its cellular anti-apoptotic functions. By regulating the activation of Akt, PTEN can also mediate the anti-apoptotic downstream effects of EGF-R signalling. Through Akt activation, EGF-R induces Bad phosphorylation, thereby inhibiting its proapoptotic interaction with Bcl-2 and Bcl-x.[58] Functional inactivation of PTEN (often loss of 1 allele followed by mutation in the other) has been observed in several human cancers.[59] In a clinical study, Frattini et al.[60] have demonstrated that PTEN loss was associated with cetuximab resistance in 27 patients with metastatic colorectal cancers. Similar data have been detailed by Jhawer et al.[61] who found that constitutive and simultaneous activation of the K-ras/B-Raf and PIK3CA pathways confers maximal resistance to cetuximab in 22 colon cancer cell lines suggesting that a priori screening of colon tumours for PTEN expression status and PIK3CA and Ras/B-Raf mutation status could help stratify patients likely to benefit from this therapy. Several other studies have provided evidence that a mutant PTEN phosphatase may lead to gefitinib resistance, suggesting that PTEN dysfunction leaves the PI3K/Akt pathway unopposed and can thereby bypass EGF-R inhibition.[47] Using a human NSCLC xenograft model, Ihle et al.[62] have shown that treatment of the mice with the PI3K inhibitor PX-866 potentiated the anti-tumour effects of EGF-R inhibitors, suggesting that PI3K inhibitors may be useful in increasing the response of gefitinib or erlotinib in patients with NSCLC and other cancers who do not respond to EGF-R inhibition.

Several other downstream signalling mediators (e.g. Akt, mTOR, src kinases, STAT proteins, K-ras and MEK1,2) have been reported to bypass EGF-R inhibition by constitutive activation of multiple pathways,[47] and some of them (mTOR and MEK1,2 inhibitors) are now being targeted in combination with EGF-R inhibitors in early-phase clinical trials. Amongst them, the ras/MAP pathway is of potential clinical interest. The ras proteins are members of a large superfamily of guanosine-5′-triphosphate (GTP)-binding proteins that play a complex role in the normal transduction of growth factor receptor-induced signals.[63] Stimulation of growth factor receptors, such as EGF-R, causes activation of multiple regulatory molecules, including the ras protein. EGF-R activates ras by stimulating its binding to GTP. Ras in its active, GTP-bound state binds several key target proteins, which results in the subsequent activation of several downstream pathways, including those mediated by MAP kinase, PI3K and others.[64] Engagement of these pathways leads to the stimulation of cell cycle progression, desensitisation of the cell to proapoptotic stimuli, changes in cytoskeletal organisation and invasion, and other processes required for cell proliferation. Activating mutations in the K-ras gene, which result in EGF-R-independent activation of the MAP kinase pathway, are found in approximately 15–30% of patients with NSCLC and in 40–45% of patients with colorectal cancer, and their presence generally correlates with a worse prognosis with respect to the outcome of the cancer. In most cases, the somatic ras missense mutations found in cancer introduce amino acid substitutions at positions 12 (Gly–>Val), 13 and 61. These mutations disable the endogenous GTPase activity of the ras protein, and cause cancer-associated ras to accumulate in the active, GTP-bound conformation. This, in turn, results in the activation of PI3K, MAP kinase and others, which results in malignant transformation. Because ras is downstream from EGF-R, aberrant ras signalling, like the one occurring in cells with mutant K-ras, may lead to dysregulation of ras-dependent pathways and downstream signalling even if the upstream receptor is silenced by anti-EGF-R monoclonal antibodies or RTKs.

In several studies, K-ras mutations have been significantly associated with lack of response to EGF-R tyrosine kinase inhibitors in patients with NSCLC and with lack of response to cetuximab or to panitumumab in patients with advanced colorectal cancer (see next paragraph). Both findings suggest that EGF-R-independent, constitutive activation of the K-ras signalling pathway could impair the response to anti-EGF-R drugs.[61]

However, other mechanisms could additionally contribute to disease progression in these patients. The discovery of somatic mutations in the tyrosine kinase domain of EGF-R in NSCLC represents a dramatic step in elucidating genomic

changes in lung cancer and their role in developing treatment strategies. These gain-of-function mutations enhance EGF-R activation, markedly increase sensitivity to EGF-R RTK inhibitors and are transforming. Retrospective studies suggest particularly promising results with EGF-R RTK inhibitors therapy among patients harbouring EGF-R mutations, with response rates higher than 65% and median survival of 20–30 months.[65] Characteristics associated with EGF-R mutations enable clinical profiling of patients to enrich for mutations among patients with NSCLC.[66] Nearly 90% of these mutations occur as either multinucleotide in-frame deletions in exon 19 or as single missense mutations that result in substitution of arginine for leucine at position 858 (L858R). Both mutations are associated with increased sensitivity to the selective EGF-R kinase inhibitors gefitinib and erlotinib.[67] About 70% of the patients with EGF-R mutations respond to EGF-R tyrosine kinase inhibitors including gefitinib and erlotinib, whereas only 10% of those without the mutations do so.[68] While most patients with EGF-R mutations derive benefit from EGF-R RTK inhibitors, there is variability in the degree and duration of response. Some patients exhibit *de novo* resistance, and the remainder are highly likely to develop acquired resistance after a period of initial response. *De novo* resistance mechanisms among patients with EGF-R mutations have not been well studied, though some genomic mechanisms of acquired resistance are recognised, including a secondary point mutation in EGF-R (T790M) that blocks the capacity for gefitinib or erlotinib to inhibit EGF-R. Mutations that substitute methionine for threonine at position 790 in the EGF-R kinase domain ('gatekeeper mutation') have been found in approximately 50% of lung adenocarcinomas from patients with acquired resistance to the EGF-R inhibitors gefitinib and erlotinib.[69–71] Threonine 790 is the 'gatekeeper' residue, an important determinant of inhibitor specificity in the ATP binding pocket. The T790M mutation has been thought to cause resistance by sterically blocking binding of tyrosine kinase inhibitors such as gefitinib and erlotinib, but this explanation is difficult to reconcile with the fact that it remains sensitive to structurally similar irreversible inhibitors (Table 3). Recently, Yun and colleagues[72] have shown that the T790M mutation activates wild-type EGF-R and that introduction of the T790M mutation increases the ATP affinity of the oncogenic L858R mutant by more than an order of magnitude. The increased ATP affinity is therefore, the primary mechanism by which the T790M mutation confers drug resistance. In addition, the authors concluded that the T790M mutation is a 'generic' resistance mutation that will reduce the potency of any ATP-competitive kinase inhibitor and that irreversible inhibitors overcome this resistance simply through covalent binding, not as a result of an alternative binding mode. Thus far, gatekeeper mutants have proved particularly difficult to be overcome in the clinic presumably because many kinase inhibitors are designed to interact with the adjacent hydrophobic (selectivity) pocket. This knowledge has led to the identification of alternative EGF-R inhibitors that can overcome T790M-mediated resistance *in vitro* and potentially in patients (Table 3).[73–75]

In contrast to the T790M mutation, in lapatinib resistance screens mutations at 16 different erbB-2 amino acid residues with 12 mutated amino acids mapping to the kinase domain were identified.[78] Mutations conferring the greatest lapatinib resistance cluster in the *N*-terminal kinase lobe and hinge region. Structural computer modelling studies suggested that lapatinib resistance is caused by multiple mechanisms; including direct steric interference and restriction of conformational flexibility (the inactive state required for lapatinib binding is energetically unfavourable). ErbB-2 T798I imparts the strongest lapatinib resistance effect[78] and is autologous to the EGF-R T790M, bcr-abl T315I and c-kit T670I gatekeeper mutations that are associated with clinical drug resistance. In contrast, data from an experimental study provided evidence that lapatinib resistance in HCT116 cells can also be mediated by elevated MCL-1 expression and by decreased BAK activation, but not by erbB receptor kinase mutations, suggesting that other mechanisms may also contribute to the observed lapatinib resistance.[79]

4. Clinical relevance of resistance

4.1. *Effect of K-ras mutation on response to anti-EGF-R therapy*

The clinical relevance of K-ras mutations has been evaluated retrospectively in several clinical trials investigating the effect of EGF-R inhibitors such as cetuximab or panitumumab in the first-line treatment of metastatic colorectal cancer. Van Cutsem and colleagues[80] reported the CRYSTAL trial in which 5-fluorouracil, folinic acid and irinotecan (FOLFIRI) plus cetuximab were compared to FOLFIRI alone. An analysis of 45% of the study population (540 of 1198 patients) revealed a K-ras mutation in 35.6% of evaluable tumours. This study demonstrated that the addition of cetuximab to FOLFIRI significantly improved PFS in K-ras-wild-type (K-ras-WT) patients (HR = 0.68, $p = 0.017$), while no improvement was observed in patients with K-ras-mutant tumours (K-ras-Mut) (HR = 1.07, $p = 0.47$). Likewise, ORR was also significantly improved in K-ras-WT patients (43% versus 59%, $p = 0.0025$), but not in the mutant population (40% versus 36%, $p = 0.46$).

Table 3 – Experimental drugs with activity in tumours harbouring the T790M mutation.

Drug	Target	Company	Status	Reference
EKB-569	EGF-R	Wyeth	Phase I	[76]
HKI-272	panErbB	Wyeth	Phase II	[74]
BIBW-2992, BIBW-2996	EGF-R, erbB-2	Boehringer–Ingelheim	Phase II	[75]
CI-1033	panErbB	Pfizer	Phase II	[66]
EXEL-7647	erbB-2, EGF-R, EphB4, VEGF-R-2	Exelixis	Phase II	[74]
CL-387,785	EGF-R	Wyeth	Pre-clinical	[77]

In the OPUS trial, a combination of 5-fluorouracil, folinic acid and oxaliplatin (FOLFOX4) was selected as a chemotherapy backbone and the addition of cetuximab was investigated in a randomised trial.[81] Of 337 patients included in this first-line trial, 233 patients could be evaluated for their K-ras status, and a K-ras mutation was found in 42%. In K-ras-WT patients, the addition of cetuximab to FOLFOX4 caused a significant increase in ORR (61% versus 37%, $p = 0.011$) and PFS (HR = 0.57, $p = 0.016$). In contrast, a negative impact on treatment efficacy was noted when cetuximab was applied in K-ras-Mut patients with regard to PFS (HR = 1.83, $p = 0.0192$) and ORR (33% versus 49%, $p = 0.106$).

A comparable effect was also noted in the aforementioned CAIRO II trial in which capecitabine/oxaliplatin (CapOx) plus bevacizumab was compared to the same regimen plus cetuximab.[57] The addition of cetuximab did not affect ORR or PFS in K-ras-WT patients. However, in K-ras-Mut patients it induced a markedly shorter duration of PFS (8.6 months versus 12.5 months, $p = 0.043$) and OS (19.2 months versus 24.9 months). An explanation for this apparently negative interaction of cetuximab with oxaliplatin-based chemotherapy in K-ras-Mut patients cannot be provided at present time.

The PACCE-trial was designed to investigate double-targeting of VEGF and EGF-R. Patients with metastatic colorectal cancer received first-line treatment with irinotecan- or oxaliplatin-based chemotherapy and were randomised to additional treatment with either bevacizumab plus panitumumab or bevacizumab alone.[82] A subgroup analysis was performed on patients with irinotecan-based chemotherapy ($n = 200$). In K-ras-WT patients ($n = 115$), the addition of panitumumab to irinotecan/bevacizumab-based therapy induced an ORR of 54% compared to 47% without the EGF-R-inhibitor. Also in this study, no improvement of ORR was observed when panitumumab was applied in K-ras mutant patients (30% versus 38%). Taken together, the PACCE study and the CAIRO II study indicate that in the presence of VEGF-inhibition by bevacizumab an additional inhibition of EGF-R does not provide further clinical benefit.

All the 4 studies, the CRYSTAL-, OPUS-, CAIRO II-, and the PACCE-trial, uniformly demonstrate that K-ras mutation confers resistance to anti-EGF-R-directed antibodies. Furthermore, these data are supported by a large body of evidence coming from phase II- and case control studies, showing the lack of efficacy of anti-EGF-R antibodies in pre-treated patients.[83–88] As a consequence, registration of cetuximab and panitumumab limits their use to patients with K-ras wild-type tumours. Determination of the K-ras mutational status is therefore, required before the clinical application of anti-EGF-R-directed antibodies. At present, multiple methods are available for the detection of K-ras mutations. Given that cross-validation of sensitivity, specificity and reliability is presently being evaluated, a single best method has not yet been defined.

Skin toxicity developing during the first weeks of the treatment is an important predictor of response to anti-EGF-R therapy,[89] but is independent of the K-ras mutational status. The greatest benefit from anti-EGF-R therapy may therefore, be expected in K-ras wild-type patients reacting to treatment with marked skin toxicity.[90]

4.2. *Effect of K-ras mutation status on response to anti-VEGF therapy*

Given that VEGF is regulated downstream of EGF-R and that inhibition of EGF-R may cause a downregulation of VEGF expression, it was of interest to investigate the effect of K-ras mutations on anti-VEGF-directed therapy.[91,92] Ince and coworkers performed a retrospective analysis of the pivotal phase III trial in which the addition of bevacizumab to the first-line therapy with irinotecan, 5-fluorouracil and leucovorin (IFL) was tested.[93,94] Microdissected tumours from 295 patients were available for the determination of mutations in K-ras (35%), B-raf (5.6%) and p53 (68%). As for overall survival, K-ras- and B-raf-wild-type patients had a better prognosis than patients with mutant tumours, but all subgroups showed a benefit from treatment with bevacizumab. In patients who were wild-type for both, K-ras and B-raf, the hazard ratio in favour of bevacizumab treatment was 0.57 (95% CI, 0.31–1.06), while it was 0.67 (95% CI, 0.37–1.20) in patients with mutant tumours. Considering the limitations of a retrospective analysis, the authors suggest that the survival benefit induced by bevacizumab was independent of K-ras-, B-raf- or TP53 mutation status.[94]

4.3. *Clinical relevance of mutation analysis in NSCLC*

There is also a growing body of evidence to support that somatic mutations in NSCLC are important to predict anti-tumour activity of EGF-R TK inhibitors (Table 4). In a recent study by Jackman et al. the impact of EGF-R and K-ras genotype on the outcomes in a clinical trial registry of NSCLC patients treated with erlotinib or gefitinib was investigated.[98]

Table 4 – High efficacy of EGF-R-tyrosine kinase inhibitors in NSCLC harbouring somatic EGF-R mutations.

Reference	Screened tumours	EGF-R-mutation	Agent	Treated patients	RR (%)	PFS (mo)
Inoue [95]	75	25 (33%)[a]	Gefitinib	16	75	9.7
Asahina [96]	82	20 (24%)[a]	Gefitinib	16	75	8.9
Sequist [65]	98	34 (35%)[b]	Gefitinib	31	55	9.2
Paz-Ares [97]	428	67 (16%)[a]	Erlotinib	43	82	13.3
Jackman [98]	205	74 (36%)[b]	Erlotinib or gefitinib	74	64	11.8[c]

RR, response rate; PFS, progression-free survival.
a del 19, L858R.
b del 19, L858R, exon 20 insertions, T790M/L858R, G719A, L861Q.
c Time to progression.

Among the 205 evaluated patients 74 had a sensitising mutation of their EGF-R without a concomitant resistance mutation. Treated with the first-line EGF-R tyrosine kinase inhibitors these patients achieved a remissions rate of 64% accompanied by a TTP of 11.8 months and a median overall survival of 23.8 months. No significant benefit was, however, observed in patients with K-ras mutations (ORR = 0%, TTP = 3.6 months, OS = 13.0 months). Moreover, patients who were wild-type for both EGF-R and K-ras had similar outcomes as those with K-ras mutations. From this analysis it may be concluded that screening for somatic EGF-R- and K-ras mutations may serve as a potent tool to select patients who will benefit from the first-line treatment with tyrosine kinase inhibitors. These findings need to be confirmed in larger sets of prospective analyses, and have not yet affected the registration status of receptor tyrosine kinase inhibitors for treatment of NSCLC.

Studies over the last few years have identified several anti-EGF-R and anti-VEGF(-R) resistance mechanisms. These findings have led to clinical trials using newly designed targeted therapies that can overcome these resistance mechanisms and have shown promise in laboratory studies. Ongoing research efforts will likely continue to identify additional resistance mechanisms, and these findings will hopefully translate into effective therapies for different cancers.

Conflict of interest statement

None declared.

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