Chapter 3
Mechanisms of Cetuximab Resistance and How to Overcome It

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer type worldwide and accounts for a standardised worldwide incidence of roughly 600,000 individuals/year [1]. HNSCC remains one of the most challenging malignancies to treat. For example, the overall 5-year relative survival proportion for the Belgian 2013–2017 cohort was about 52% in males and 59% in females [2]. Unfortunately, patients with multiple metastases typically have very poor prognosis with a 5-year overall survival of only 4% [3]. Therefore, innovative therapeutic strategies are a necessity to increase the survival outcomes.

The introduction of targeted therapies that inhibit oncogenic signalling pathways is now at the forefront of personalised medicine in cancer treatment. As the epidermal growth factor receptor (EGFR) initiates important signalling pathways and is overexpressed and/or deregulated in a wide range of malignancies, this receptor is considered as an excellent drug target. Improved understanding of EGFR signalling in cancer has led to the development of two main categories of EGFR-targeting agents: the monoclonal antibodies (mAbs, such as cetuximab and panitumumab) and the tyrosine kinase inhibitors (TKIs, such as erlotinib and gefitinib) [4].

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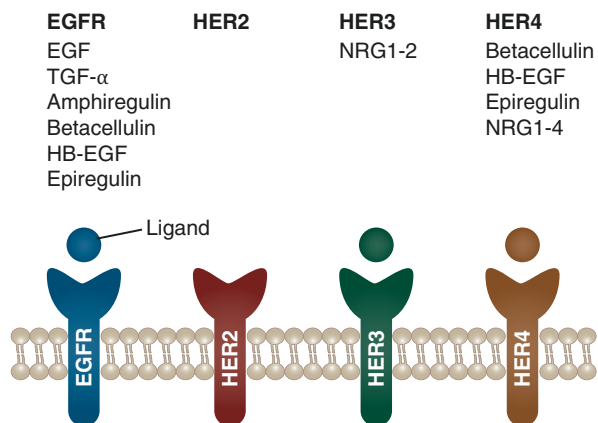e-mail: An.Wouters@uantwerpen.be
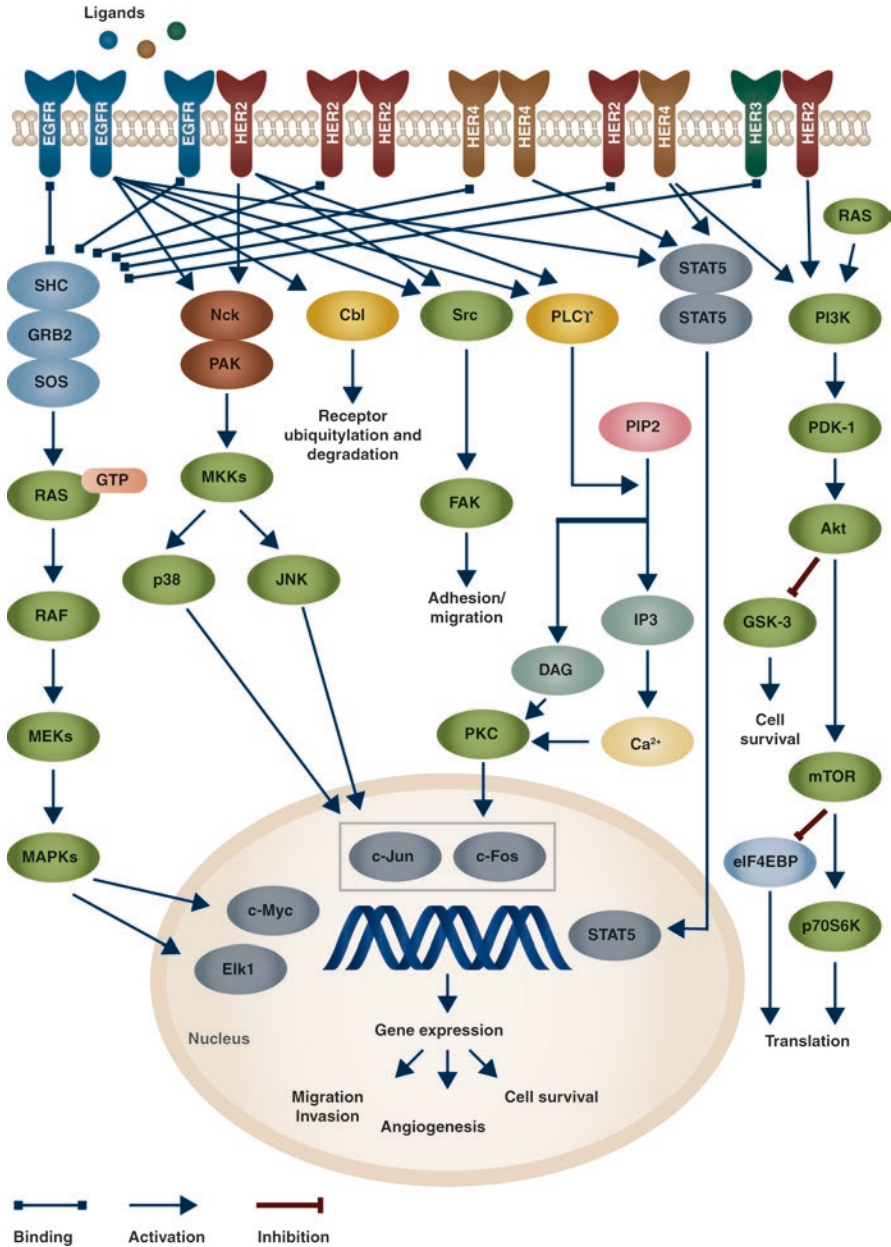
The Epidermal Growth Factor Receptor

The physiological function of EGFR is to regulate epithelial tissue development and homeostasis through cellular processes such as proliferation, maturation and apoptosis [5]. These cellular processes play an important role in the transformation of healthy cells into malignant tumour cells. The activity of the EGFR signal transduction pathway is tightly controlled in healthy cells, but deregulation of EGFR signalling plays a crucial role in carcinogenesis [6]. Accordingly, EGFR signalling has been studied intensively in order to understand its importance in cancer biology.

EGFR is a cell surface receptor that belongs to the HER or ErbB tyrosine kinases family. Besides EGFR, also known as HER1 or ErbB1, other members of the HER family include HER2 (ErbB2 or Neu), HER3 (ErbB3), and HER4 (ErbB4). The structure among these receptors is very similar: they consist of an extracellular ligand-binding domain, a single membrane-spanning region, a juxtamembrane nuclear localisation signal and a cytoplasmic tyrosine-kinase domain [7]. HER receptors are activated by a range of growth factors that belong to the EGF-family and can be divided into three groups (Fig. 3.1). The first group includes EGF, transforming growth factor-α (TGF-α) and amphiregulin, which all bind to EGFR. The second group includes betacellulin, heparin-binding EGF (HB-EGF) and epiregulin which bind to both EGFR and HER4. The third group is composed of the neuregulins (NRG1-4), which is further subdivided based on their ability to bind HER3 and HER4 (NRG1 and NRG2), or only to HER4 (NRG3 and NRG4) [8]. Until now, no known ligand exists for HER2 [7, 9].

Binding of a ligand to the extracellular domain of these receptors leads to a conformational change that allows for receptor homo- and hetero-dimerisation and activation of intrinsic tyrosine kinase activity resulting in transphosphorylation of specific tyrosine residues within the intracellular domain. Autophosphorylation triggers a series of intracellular pathways that may result in cancer-cell proliferation, blocking apoptosis, activating invasion and metastasis, and stimulating tumour-induced neovascularisation. Figure 3.2 gives an overview of the HER receptor

Fig. 3.1 The HER family receptors and ligands. Binding of a ligand to the extracellular domain of the receptor leads to receptor homo- and hetero-dimerisation and activation. TGF-α, transforming growth factor-α; HB-EGF, heparin-binding EGF

EGFR
EGF
TGF-α
Amphiregulin
Betacellulin
HB-EGF
Epiregulin

HER2

HER3
NRG1-2

HER4
Betacellulin
HB-EGF
Epiregulin
NRG1-4

Ligand

EGFR   HER2   HER3   HER4

Fig. 3.2 HER receptor signalling pathways. Ligand binding to HER receptors is followed by receptor homo- and hetero-dimerisation and the activation of several downstream signalling pathways

signalling pathways, including the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, the signal transducers and activators of transcription (STAT) pathway, Phospholipase Cγ, Src kinase pathways, the NcK/PAK signalling cascade and Cbl-mediated endocytosis.

During the 1980s, several studies described the overexpression of EGFR in a variety of epithelial tumours, which supported the hypothesis that dysregulated EGFR expression and signalling play an important role in the development of cancer [7, 10–14]. Over the last years, the oncogenic role of EGFR has been characterized in more detail and several alterations have been described [15]. Firstly, gene amplification leading to EGFR overexpression is often observed in human cancers [16, 17]. Secondly, point mutations and deletions in the EGFR gene can result in increased catalytic tyrosine kinase activity or a truncated form of the receptor, resulting in ligand-independent activity [18]. The most common tyrosine kinase EGFR mutations include the deletion of four conserved amino acids residues in exon 19 and the point mutation L858R in exon 21 [19, 20]. In addition, the EGFR variant III (EGFRvIII) is also frequently detected and constitutes a truncated form of EGFR caused by an in-frame deletion of 801 base pairs (exon 2–7) in the coding sequence of the extracellular domain [21, 22]. Next, increased ligand expression leads to constitutive stimulation of EGFR. EGF-related growth factors can be produced either by the tumour cells themselves or by surrounding stromal cells [23]. Finally, impaired receptor downregulation also results in sustained EGFR signalling [24]. All these alterations, consequently, result in increased EGFR activation and/or deregulation of EGFR signal transduction pathways. As EGFR stimulates many complex intracellular signalling pathways that are involved in proliferation, differentiation, apoptosis, angiogenesis and metastasis, activation of EGFR drives the malignant behaviour of the tumour [25].

Cetuximab as Anti-EGFR Targeting Agent

For over a decade, the EGFR monoclonal antibody cetuximab is approved by the European Medicines Agency (EMA) and Food and Drug Administration (FDA) for HNSCC treatment in three specific settings, i.e. (1) in combination with radiation therapy for locoregionally advanced HNSCC; (2) in combination with platinum-based therapy and 5-fluorouracil for first-line treatment for recurrent or metastatic (R/M) HNSCC; and (3) as a single agent for R/M HNSCC patients who failed on prior platinum-based therapy [26–28].

The therapeutic effect of the monoclonal antibodies, such as cetuximab and panitumumab, is exerted by binding to the extracellular domain of EGFR, thereby hindering ligands to bind and activate EGFR, preventing receptor dimerisation and promoting EGFR internalisation [15]. Importantly, as a chimeric human:mouse immunoglobulin G1 (IgG1), cetuximab can also elicit host anti-tumour immune responses. Through its IgG1 backbone, cetuximab can bind CD16 fragment

crystallisable (Fc) receptors located on natural killer (NK) cells, macrophages and granulocytes, of which NK cells have been proven to be the most potent effectors [29]. Binding of the IgG1-Fc part of cetuximab to CD16 on NK cells triggers cytolytic activity called antibody-dependent cellular cytotoxicity (ADCC), which is predominantly mediated by perforin and granzymes [30]. Furthermore, cetuximab has been shown to enhance cross-priming of cytotoxic T-lymphocytes via professional antigen-presenting cells, such as dendritic cells [31], mainly through induction of immunogenic cell death of tumour cells [32]. These results confirmed the important immune-related mechanism of action of cetuximab, in addition to its receptor blocking effects.

Mechanisms of Cetuximab Resistance

Personalised medicine using targeted therapies, based on the molecular profile of the tumour, may achieve the much-needed progress in cancer treatment. After the initially promising results of EGFR-targeted therapies such as cetuximab, therapeutic resistance poses a challenging problem and limits the success of effective cancer therapies in the clinic [33]. If resistance to therapy is present at baseline, this is defined as intrinsic (primary) resistance and can be explained by resistance-conferring factors pre-existing in the bulk of tumour cells. Moreover, nearly all patients whose tumours initially respond inevitably become acquired (secondary) resistant. Acquired resistance refers to disease progression in the face of ongoing treatment that was initially effective [34].

Indeed, despite the enhanced EGFR expression in the majority of HNSCC tumours, therapeutic resistance remains a major roadblock in the search to effective HNSCC therapies and only a small subset of HNSCC patients benefit from cetuximab as a single agent (<15%) or combined with chemotherapy (36%). The addition of cetuximab to either radiotherapy in the locoregionally advanced disease setting, or to platinum and 5-fluorouracil chemotherapy (EXTREME regimen) for treatment of recurrent or metastatic HNSCC improved median overall survival from 29.3 to 49 months and from 7.4 to 10.1 months, respectively. Nevertheless, time-to-treatment failure in patients treated with the EXTREME regimen ranges only around 5 months, despite cetuximab maintenance [28, 35, 36].

Therapeutic resistance to anti-EGFR therapy may arise from mechanisms that can compensate for reduced EGFR signalling and/or mechanisms that can modulate EGFR-dependent signalling. Over the last years, a wide range of potential molecular mechanisms of resistance to EGFR-targeting agents has been described [15].

Altered Response Elicited at the Level of EGFR

Drug resistance can arise from sustained EGFR signalling that is elicited at the level of EGFR itself by ligand or receptor overexpression, amplification or mutation [15, 19, 20, 37–41]. Furthermore, EGFR can escape the internalisation and lysosomal degradation route and function as a transcription factor in the nucleus, inducing sustained EGFR signalling [42].

Binding of ligands to EGFR drives homodimerisation or heterodimerisation with ErbB family members, resulting in the initiation of downstream signalling pathways. Therefore, overexpression of its ligands may contribute to cetuximab resistance. A correlation with enhanced response to cetuximab therapy and overexpression of the EGFR ligands amphiregulin and epiregulin in K-Ras wild type metastatic colorectal tumours has been reported [40]. In HNSCC patients receiving cetuximab-docetaxel treatment, high amphiregulin levels were detected in 45% of the patients. A significant correlation was found between high amphiregulin levels and shortened overall survival and progression free survival compared with patients with low amphiregulin expression [41].

Neither the expression level of the EGFR protein, nor the amplification status of the *EGFR* gene could be linked to therapeutic response [43, 44]. Activating mutations have been observed in the tyrosine kinase domain or in the extracellular ligand-binding domain of EGFR [18]. The most common tyrosine kinase EGFR mutations include deletion of four conserved amino acids residues (leucine-arginine-glutamic acid-alanine) in exon 19 and a point mutation, L858R, in exon 21, which account for 90% of all EGFR tyrosine kinase mutations in non-small cell lung cancer (NSCLC) [45–47]. These EGFR tyrosine kinase mutations are associated with an improved clinical response to TKIs (gefitinib or erlotinib) in NSCLC patients but they are rarely found in HNSCC. Literature data suggest that the incidence of such activating mutations in HNSCC patients range from 0 to 15.7% [45–60]. In these studies, a total of 889 HNSCC samples were screened for EGFR tyrosine kinase mutations, of which 34 (3.8%) contained a mutation. Interestingly, the missense mutation T790M in exon 20, which is associated with acquired resistance to EGFR TKIs in about half of all patients with NSCLC, was found in 7.5% of all EGFR mutations in HNSCC [61]. Given that in HNSCC, the overall prevalence of mutations in the EGFR tyrosine kinase domain is only 2.8%, it is challenging to identify specific EGFR mutations related to response or resistance to anti-EGFR therapy [62].

Next to the above-discussed mutations, the EGFR variant III (EGFRvIII) is a truncated form of EGFR. The causing mutation consists of an in-frame deletion of 801 base pairs (exon 2–7) in the coding sequence of the extracellular domain, resulting in ligand-independent tyrosine kinase activity [21, 63]. The mutant EGFRvIII form is associated with increased proliferation, tumour growth, cell motility and invasion in vitro and resistance to anti-EGFR therapy [21, 64]. The mutation frequency of EGFRvIII in HNSCC ranges from 0 till up to 48% [41, 49, 51, 64–67]. It has been suggested that the EGFRvIII might be more available in the recurrent/

metastatic disease setting and might be responsible for the lack of response to EGFR-targeted therapies [67].

Occasionally, a part of the EGFR receptor escapes the internalisation and lysosomal degradation route and translocates to the nucleus [42, 68]. In oral squamous cancers, nuclear EGFR was observed in 24.3% of patients [69]. This nuclear EGFR functions either as a transcription factor of cyclin D1, inducible Nitric Oxide Synthase (iNOS), B-Myb and cyclo-oxygenase-2 (COX-2), or as a tyrosine kinase phosphorylating and stabilizing proliferating cell nuclear antigen (PCNA), resulting in an activation of the nitric oxide pathway and increased G_1/S progression of the cell cycle [70–74]. Consequently, the proliferative potential of the cancer cells is thereby enhanced. The presence of nuclear EGFR is not only associated with poor prognosis, but also with treatment resistance [69, 75, 76]. Besides its potential involvement in resistance mechanisms, nuclear EGFR is also associated with local recurrence [75].

Molecular Alterations in Effectors Downstream of EGFR

Resistance to EGFR inhibitors can also be the result of molecular alterations in effectors downstream of EGFR [15]. In particular, the RAS, PI3K, Akt, STAT and Src proteins have been suggested to contribute to drug resistance [77–88]. We previously showed that proteins related to the Ras-MAPK pathway are involved in mechanisms of resistance towards cetuximab in HNSCC [89]. This is confirmed in other studies, highlighting the significance of persistent activation or reactivation of the Ras-MAPK pathway in EGFR targeting drug resistance [90–92].

Firstly, K-Ras is a protein located downstream of EGFR in the Ras-MAPK pathway. Somatic point mutations in *K-Ras* occur in a variety of human malignancies, most frequently in pancreatic cancer, NSCLC and colon cancers [93, 94]. A mutation in codon 12 or 13 in this gene leads to constitutive activation of the protein, regardless of upstream activating signals. In colorectal tumours, these mutations confer resistance to therapy with the EGFR targeting monoclonal antibodies cetuximab and/or panitumumab [77, 78, 95, 96]. Approximately 30–40% of colorectal tumours harbour a *K-Ras* mutation [97, 98]. In contrast, in HNSCC, these *K-Ras* mutations are infrequent; in different reports the frequency of *K-Ras* mutations in HNSCC is ranging from 0 to 9.1% [45, 51, 99].

Another family member of the Ras proto-oncogenes is H-Ras. Mutations in *H-Ras* have been reported in literature and vary between 0 and 22% [100–104]. A very recent study demonstrated that *KRAS/HRAS* mutations were associated with poor progression-free survival among HNSCC patients treated with cetuximab in the first-line recurrent setting, but not among patients treated with cetuximab in combination with radiotherapy [105], thus suggesting that not only *K-Ras* but also *H-Ras* mutations might play a role in cetuximab resistance.

Secondly, further downstream of K-Ras in the MAPK signalling pathway, a member of the dual-specificity phosphatase (DUSP) family is located. DUSP

proteins are involved in a negative feedback mechanism of the MAPK signalling pathway by dephosphorylation of the threonine-glutamic acid-tyrosine motif on MAP kinases [106]. Therefore, DUSP proteins can be seen as tumour suppressor proteins, and loss of their expression may promote constitutive activation of ERK and uncontrolled cell growth. Moreover, inhibition of the MAPK pathway can be compensated by suppression of the DUSP enzymes [92]. Both the cytoplasmic DUSP5 and the nuclear DUSP6 can dephosphorylate ERK1/2, thereby blocking the MAPK signal transduction cascade [107].

Low DUSP6 expression has proven to be clinically significant as it was observed in 40% of patients with oesophageal squamous cell carcinomas and 75% of nasopharyngeal patients [108]. This might occur through hypermethylation of CpG islands in intron 1 or loss of heterozygosity of the DUSP6 locus [109, 110]. As DUSP6 is a critical negative regulator of Erk1/2 [111], we previously evaluated the level of Erk1/2 phosphorylation and demonstrated that significantly more Erk1/2 phosphorylation was present in cetuximab resistant HNSCC cells after cetuximab treatment compared with cetuximab sensitive HNSCC cells. Furthermore, apigenin, an Erk1/2 inhibitor, dose-dependently inhibited survival of cetuximab resistant cells and a significant decrease in cell survival was observed when these cells were treated with a combination of apigenin and cetuximab [89]. Additionally, sustained or reactivated Erk, caused by downregulation of *DUSP6*, has been observed in lung cancer cells with acquired erlotinib resistance [91]. This highlights the significance of our findings and indicates that the exact function of the DUSP family proteins in relation to cetuximab resistance in HNSCC should be further elucidated.

Thirdly, besides activation of the Ras/Raf/MAPK signalling pathway, EGFR can also mediate activation of the PI3K/Akt pathway. This pathway is involved in various biological processes essential for normal cellular functionality, including survival, proliferation, differentiation, angiogenesis, protein synthesis and glucose metabolism. Besides these physiological functions, the pathway is also associated with a number of oncogenic processes and is one of the most frequently dysregulated pathways in cancer, including HNSCC [112, 113]. As such, aberrant signalling can lead to the stimulation of cell growth, inhibition of cell death and the promotion of invasion and migration [114–116], which is all in favour of cancer cells. Furthermore, increasing evidence indicates that the PI3K/Akt pathway frequently remains activated, despite anti-EGFR treatment and therefore plays an important role in resistance to EGFR-targeting therapies [117–120].

Fourthly, Src kinases are upstream as well as downstream activators of EGFR and other receptor tyrosine kinases. Upon EGFR stimulation, Src kinases are activated and associate with EGFR. As such, they can affect cellular proliferation and survival by activation of STAT family of transcription factors, especially STAT3 and STAT5 [121, 122]. In vitro studies showed reduced activity of Src kinases following EGFR inhibition [123]. Elevated Src levels and/or kinase activity have been shown in HNSCC and other malignancies [122, 124]. Therefore, activation of Src kinases by EGFR upstream or downstream signalling might result in resistance to anti-EGFR therapy.

As mentioned earlier, nuclear translocation of EGFR is a possible mechanism of resistance to therapy and this has been observed in patients treated with cetuximab and radiotherapy. Phosphorylation of EGFR on tyrosine 845 by the Src kinases enhances EGFR-mediated mitogenesis by binding and phosphorylating the STAT5b transcription factor and this has been described as the underlying mechanism responsible for nuclear translocation of EGFR [88, 125]. Indeed, dasatinib, a Src inhibitor, blocks EGFR translocation to the nucleus in HNSCC cell lines and, therefore, might be a potential way to evade resistance to anti-EGFR therapy [126]. In addition, in oral squamous cell carcinoma, it was shown that the combination of cetuximab and a Src inhibitor may provide more effective therapy than either inhibitor alone [127]. Collectively, these results indicate that Src inhibitors may be useful in overcoming anti-EGFR resistance by decreasing activated STAT3 and STAT5.

Finally, when considering resistance to anti-EGFR therapy, the signal transducer and activator of transcription (STAT) family proteins are also important downstream EGFR effectors. This family plays an important role in transmitting survival signals and anti-apoptotic signals that are initiated through activation of EGFR; especially activation of STAT3 and STAT5 has been linked to phosphorylation of EGFR [122, 128, 129]. Therefore, dysregulation of the STAT signalling pathway has been proposed to be implicated in malignant transformation.

Activation of STAT3 leads to the activation of several survival proteins, including Bcl-xl, Bcl-2 and survivin [84]. In HNSCC, STAT3 activation can be mediated by JAK and Src signalling, and partially by EGFR signalling [126, 130]. As such, STAT3 can be inhibited via EGFR blocking and this has been demonstrated in vitro and in vivo [131]. It has been shown that the anti-proliferative effects of cetuximab, as well as cetuximab-induced apoptosis, are more pronounced in STAT3 knockdown cells compared to control cells [84]. Recently, increased STAT3 expression was found in two acquired cetuximab-resistant HNSCC cell lines, compared to their parental lines. Moreover, STAT3 knockdown promoted increased cytotoxicity both in the presence and absence of cetuximab in the resistant lines [132], suggesting that STAT3 may be a common target in cetuximab resistance.

Cross-talk with Other Receptor Tyrosine Kinases

Selective stress of EGFR-targeting agents can lead to activation of alternative signalling pathways to compensate for the reduced EGFR signalling, thereby promoting cell survival [15]. Examples of alternative receptor pathways include other HER receptor family members, insulin growth factor type 1 receptor (IGF-1R) and MET [133–139].

Firstly, as mentioned earlier, EGFR is a family member of the ErbB receptor family, and activation of other members of this family might result in resistance to anti-EGFR therapy. In the literature, activation of HER2 signalling has been associated with cetuximab resistance, as its signalling occurs through many of the same downstream effectors of EGFR. Using an in vitro model of acquired cetuximab

resistance, a marked increase in the phosphorylation status of the C-terminal fragment of HER2, 611-CTF, was observed. Combination therapy of afatinib (an irreversible EGFR/HER2/HER4 inhibitor) with cetuximab resulted in a dramatic reduction in cetuximab resistant tumour volumes compared to either agent alone in monotherapy [133]. Therefore, it was suggested that dual inhibition of EGFR and HER2 could be an effective approach to enhance the efficacy of cetuximab, in order to prevent and/or overcome cetuximab resistance. Likewise, a study by Yonesaka et al. has shown that cetuximab resistance could be induced by activation of ErbB2 signalling. The underlying mechanism involved amplification of ErbB2 or upregulation of heregulin, both leading to persistent ERK1/2 activation. Moreover, restoring cetuximab sensitivity was accomplished by inhibition of ErbB2 or by disruption of ErbB2/ErbB3 heterodimerisation in vitro as well as in vivo [134]. More studies are warranted in order to determine the frequency of HER2 mutations in HNSCC and their role in the response to TKIs.

Secondly, activation of the insulin growth factor type 1 receptor (IGF-1R) leads to downstream activation of the Ras/Raf/MAPK and PI3K/Akt pathway and enhances survivin expression, all contributing to cell proliferation, altered cell adhesion, enhanced motility properties and impaired apoptosis [140, 141]. Analysis of the HNSCC subsets of the Cancer Genome Atlas has identified 4% amplification and mutation of IGF-1R gene in human papillomavirus (HPV) negative HNSCC patients [142]. Furthermore, activation of IGF-1R has been reported to induce resistance to EGFR TKIs [143]. It was shown that heterodimerization of EGFR with IGF-1R was increased in cetuximab resistant HNSCC cancer cells [144]. This heterodimerization of EGFR with IGF-1R lead to increased activity of EGFR and might be an important platform for cetuximab-mediated signalling in head and neck tumours that have become resistant to anti-EGFR therapy. As such, dual targeting of EGFR and IGF-1R could be a promising therapeutic strategy.

Thirdly, the *MET* proto-oncogene encodes a transmembrane receptor tyrosine kinase MET, also known as c-MET or hepatocyte growth factor receptor (HGFR). The MET pathway can be deregulated in two different ways: on the one hand by mutation and/or amplification of MET, and on the other hand by increased ligand expression and/or activity, both resulting in persistent activation of the PI3K/Akt signalling pathway [138]. Circa 80% of primary HNSCC tumours express the ligand hepatocyte growth factor (HGF), MET, or both, thus activating important downstream signals, which overlap with EGFR signalling [65, 145]. Moreover, MET mutations or amplifications have been observed in 13.5% and 13% of HNSCC tumours, respectively [146]. As high MET expression could be observed in 58% of patients with recurrent/metastatic HNSCC [65], the role of MET in resistance to anti-EGFR therapy has been investigated in a number of studies. Chau et al. did not detect any association between response to erlotinib and time to progression or overall survival, in recurrent/metastatic HNSCC patients with high MET expression [65]. Experiments in vitro and in vivo showed that MET confers resistance to cetuximab via activation of the MAPK pathway. In addition to the direct role of MET in reactivation of the MAPK pathway, MET stimulation also abrogated the well-known cetuximab-induced compensatory feedback loop of HER2/HER3 expression

[147]. In a HNSCC xenograft model, a delay in tumour growth was observed after administration of crizotinib, a MET TKI [145]. Collectively, these data suggest that high MET expression might play a role in cetuximab resistance.

Alterations in Proteins Outside the EGFR Pathway

Not only alterations in proteins involved in EGFR signalling but also proteins such as cyclin D1 and p53, linked to more general characteristics of cancer (such as proliferation, apoptosis, invasion and metastasis) can confer resistance to EGFR inhibitors [148–151].

The Aurora kinases A and B are highly conserved serine/threonine kinases that play an essential and distinct role in mitosis [152, 153]. Overexpression of both kinases is frequently present in many types of malignant tumours, and in the case of HNSCC, overexpression of Aurora kinase A is found in up to 90% of tumours [153–155]. Overexpression of Aurora kinase A is correlated with tumour progression, a metastatic phenotype and shortened survival, and is therefore regarded as a negative prognostic marker [152, 154, 155]. High expression levels of Aurora kinase B are found in glioblastoma, ovarian carcinoma and hepatocellular carcinoma and are associated with poor prognosis [156].

The EGFR pathway can elicit overexpression of Aurora kinase A at two different levels, i.e. (i) EGF increases the translational efficiency of Aurora kinase A; and (ii) translocation of EGFR to the nucleus results in binding to the Aurora kinase A promoter and thereby increasing its transcription. Both ultimately result in chromosome instability and tumourigenesis [73, 157].

Next to its role as a prognostic factor, studies indicated evidence for a role of Aurora kinase A in the response to therapy. Overexpression of Aurora kinase A triggered the activation of two important molecules involved in the regulation of drug resistance, Akt and NF-κB [158]. Interestingly, knockdown of Aurora kinase A in HeLa cells resulted in sensitisation to cisplatin, and Aurora kinase A overexpression could overcome cell death induced by paclitaxel [158]. Furthermore, treatment of HNSCC cells with cetuximab and a pan-Aurora kinase inhibitor R763 resulted in a rapid and efficient decrease in the level of the Aurora kinase substrate S10HH3. These results could not be confirmed by using a specific Aurora kinase A inhibitor and, therefore, it was concluded that the effects of the pan-Aurora kinase inhibitor were most likely mediated by its blockage of Aurora kinase B activity [152]. Similarly, we previously showed that cell growth of cetuximab resistant cells could be inhibited by blocking Aurora kinase B [89]. Collectively, these results indicate that the Aurora kinases may be an interesting target for HNSCC tumours resistant to anti-EGFR therapy.

The G_1/S-specific cyclin D1 forms a complex with CDK4 and CDK6 and functions as a regulatory subunit of CDK4 and CDK6, the activity of which is required for cell cycle G_1/S transition. As previously mentioned, nuclear EGFR functions as a transcription factor for cyclin D1. Moreover, constitutive activation of STAT3 is

required for EGFR-mediated cell growth and results in elevated levels of STAT3 target genes, including cyclin D1 [129, 159].

HNSCCs that are unrelated to the human papillomavirus (HPV), are often driven by p16[INK4A] inactivation and cyclin D1 overexpression that cause hyperactivation of cyclin-dependent kinase 4/6 (CDK4/6), which drives the cell cycle and tumour growth. Deregulated cyclin D1 expression also causes resistance to EGFR inhibitors. These somatic genomic alterations pointed to inhibition ofCDK4/6 as a potential targeted therapeutic strategy in HPV-unrelated HNSCC. The CDK4/6 inhibitor palbociclib arrests cell cycle progression by selective CDK4/6 inhibition and might also reverse intrinsic resistance to cetuximab by countering the actions of deregulated cyclin D1. The antiproliferative and antitumour effects of selective CDK4/6 inhibition have indeed been demonstrated in HNSCC cell lines and xenografts. In HPV-unrelated HNSCC cell lines, the combination of palbociclib and an EGFR inhibitor synergistically reduced cell viability andERK1/2 phosphorylation. Importantly, a recent multicentre, phase 2 trial, showed that the combination of palbociclib and cetuximab exhibited substantial antitumour activity in platinum-resistant and in cetuximab-resistant HPV-unrelated HNSCC [160]. As such, further investigation of selective CDK4/6 inhibition as a therapeutic strategy in HPV-unrelated HNSCC is certainly warranted.

The tumour suppressor protein p53 has a critical role in controlling cell cycle progression and, consequently, loss of its function is linked to the carcinogenic process. In response to a variety of cellular stimuli, p53 can induce cell cycle arrest, apoptosis or senescence.

A study investigating the difference between cetuximab resistant and their sensitive parental lung cancer cells, identified p53 as the most downregulated and pERK1/2 as the most upregulated cellular signalling protein. Downregulation of p53 was also observed in erlotinib resistant cells. Furthermore, silencing of p53 in cetuximab sensitive cells resulted in reduced sensitivity to the drug, whereas restoring p53 function in resistant cells resulted in enhanced cetuximab sensitivity [149]. In vivo experiments, using a stable cetuximab resistant clone with tetracycline-inducible p53 showed that repair of p53 restored cetuximab sensitivity in tumour xenografts resistant to cetuximab [149]. In addition, cetuximab was able to inhibit cell growth in p53 wild type cells, but not in p53 mutated cells [151]. In general, there is insufficient experimental evidence to unequivocally state that loss of functional p53 can be predictive of resistance to anti-EGFR therapy.

Epithelial-to-Mesenchymal Transition

We and others have proposed epithelial-to-mesenchymal transition (EMT) as a mechanism of resistance towards EGFR targeting therapeutics [161–169]. EMT is characterized by loss of epithelial cell characteristics and acquisition of mesenchymal phenotypic traits, causing tumour cells to detach from neighbouring cells and to migrate into adjacent tissue [170–172]. However, it has been reported that EGFR

inhibition can promote an infiltrative front composed of mesenchymal-like cells, which made up a small subpopulation of the tumour before therapy [173]. Increased expression of IL8 and HB-EGF have been linked with EMT [37, 174–180] and we showed that both genes were upregulated in our cetuximab resistant HNSCC cells and these cells shows traits of EMT, including higher migratory and invasive capacity. Moreover, our microarray profile revealed upregulation of several epithelial markers in cetuximab sensitive HNSCC cells, whereas cetuximab resistant cells were characterized by upregulation of protease urokinase (*PLAU*), transgelin (*TAGLN*), *ADAM19* and thrombospondin (*TSP-1*), all of which have functions associated with features of EMT [181–185]. Similarly, it has been reported that HNSCC cells with a mesenchymal-like morphology and elevated migratory potential were found to be less sensitive to irradiation and cetuximab [186]. Overall, these findings clearly indicate that cetuximab resistant cells show enhanced characteristics of EMT.

Hypoxia and Angiogenesis

Regions within solid tumours often experience mild to severe oxygen deprivation (hypoxia) and it has been well documented that poor oxygenation is a pathophysiological property of the majority of human solid tumours, including HNSCC [187]. Importantly, oxygen deficiency has a major impact on clinical responses to cancer treatment, and it was shown that hypoxic tumour regions often contain viable cells that are intrinsically more resistant to treatment with radiotherapy and/or chemotherapy [188, 189]. Both preclinical and clinical studies support an important link between hypoxia and upregulation of EGFR in cancers that do not display genetic alterations of the receptor [190]. Subsequent EGFR signalling stimulates hypoxia-inducible factor (HIF) signalling and thus augments induction of proteins that promote cellular survival in a hostile microenvironment. As the HIF transcription factors play a pivotal role in the cellular adaptation to hypoxic stress, EGFR-induced HIF signalling thus augments the induction of proteins that promote cellular survival in a hostile microenvironment. As a consequence, the presence of tumour hypoxia may contribute to resistance to EGFR inhibitors [191]. HNSCC patients with high levels of hypoxia-associated factors indeed were more likely to relapse, following induction therapy that included cetuximab [192], suggesting that the role of tumour hypoxia in therapeutic resistance might be particularly relevant for regimens containing EGFR-targeting monoclonal antibodies [192].

Lee et al. reported only minimal distribution of cetuximab to hypoxic tumour regions [193]. As monoclonal antibodies are large molecules, which are consumed by binding to receptors on the cell surface, this might indeed lead to poor penetration within solid tumours. However, cetuximab has a long half-life in the circulation, so that a more uniform distribution in tissues might be established, even if penetration of tissue is relatively slow. Indeed, in contradiction to the observation by Lee et al., Santiago et al. reported that cetuximab was homogeneously distributed within FaDu HNSCC xenografts, with no difference between hypoxic and

non-hypoxic tumour cells [194]. These findings were in line with clinical data on the distribution of anti-EGFR antibodies in HNSCC [195] and indicate that cetuximab accesses not only (oxygenated) cells in proximity to the tumour blood vessels, but indiscriminately reaches all tumour cells.

Only few papers illustrate hypoxia-induced treatment resistance and most studies on EGFR-targeting antibodies supported a markedly increased anti-tumour potency of cetuximab in vivo (over that observed in vitro), suggesting that factors of the tumour microenvironment might influence the in vivo response.

The first reports on this topic addressed the association between the EGFR pathway and tumour angiogenesis. Together with the demonstrated antiproliferative and pro-apoptotic effects, the anti-angiogenic activity of cetuximab is now believed to contribute to its overall anti-tumour activity in vivo. With regard to this anti-angiogenic effect, numerous studies have shown that treatment of human cancer cells in vitro and in vivo with cetuximab reduced the production of VEGF [196–198]. Luwor et al. found that cetuximab reduced the levels of HIF-1α, leading to transcriptional inhibition of VEGF expression [199]. Immunohistochemical analysis of HNSCC tumour xenografts after systemic administration of cetuximab demonstrated inhibition of the in vivo expression of tumour angiogenesis markers, including VEGF and Factor VIII [200].

Apart from the observed anti-angiogenic effects, it has also been speculated that hypoxia enhances the sensitivity to the cytotoxic effect of EGFR-targeted monoclonal antibodies [201]. For example, cetuximab was more cytotoxic against hypoxic than well-oxygenated A431 epidermoid cancer cells grown in vitro and it reduced the overexpression of hypoxia markers (HIF-1α, CA9, VEGF) [198]. Likewise, we observed that both EGFR-inhibitors cetuximab and erlotinib maintained their growth inhibitory effect under hypoxia in vitro in three cetuximab-sensitive HNSCC cell lines [201]. Whether this was a direct interaction between hypoxia- and EGFR-mediated signalling pathways or indirectly via reoxygenation as a consequence of cell loss due to the cytotoxic effect of cetuximab [202] was not elucidated in these studies, but both mechanisms might be involved.

Therefore, several studies have focused on the molecular mechanisms behind the cross-talk between hypoxia and EGFR inhibition and on the role of HIF-1α in this process [191]. Importantly, it was observed that cetuximab could clearly downregulate HIF-1α levels in cancer cell lines that were sensitive to EGFR inhibition and it was shown that inhibition of HIF-1α was required, although it might not be sufficient, to mediate the response of cancer cells to EGFR-targeted monoclonal antibodies [199, 203–205]. In contrast, overexpression of HIF-1α in cancer cells that were originally sensitive to treatment with cetuximab conferred substantial resistance to this anti-EGFR therapy [204]. It was also reported that cetuximab sensitised HNSCC cells to radiation in part through inhibition of the radiation-induced upregulation of HIF-1α [206]. Overall, further in-depth studies are needed to fully understand these observations.

As inhibition of proteasomal degradation did not alter the rate of HIF-1α reduction by cetuximab treatment, it was suggested that cetuximab mainly acts by inhibiting HIF-1α protein synthesis [199, 207]. In hypoxic gastric cancer cells, it was

shown that cetuximab reduced HIF-1α expression via inhibition of both MAPK and PI3K/AKT signalling downstream of EGFR [208]. However, most other studies suggested that the exact mechanism of reducing HIF-1α synthesis by cetuximab involved only inhibition of the PI3K/AKT pathway. The inhibition was shown to be prevented in cancer cells transfected with constitutively active PI3K or constitutively active AKT, but not in cells with a constitutively active MEK [204].

Overall, despite their individual key roles in promoting cancer progression and treatment resistance, our knowledge about the impact of intratumoural hypoxia on the activity of the EGFR signalling pathway in cancer and vice versa remains rather limited. As such, further studies are warranted to define the precise mechanistic and therapeutic implications of the hypoxic response relative to the EGFR signalling pathway in cancer.

Strategies to Overcome Cetuximab Resistance

Despite the reported intrinsic and acquired resistance to EGFR-targeting agents, interest in targeting EGFR for the treatment of HNSCC remains high, with new strategies, such as inhibitor combinations and novel irreversible or multi-targeting inhibitors, currently being evaluated.

Irreversible and Multiple HER Receptor Inhibition to Overcome Resistance

The ongoing challenge of therapy resistance has prompted a new approach to treat cancer patients, notably multiple inhibition of HER receptors simultaneously or irreversible inhibition. As mentioned above, the HER family of receptor tyrosine kinases comprises four members, i.e. EGFR (HER1, ErbB1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). The particular mode of activation of the HER network involving ligand-induced homo- and hetero-dimerisation of the four HER receptors has prompted a new approach to inhibit this complex network and prevent premature emergence of resistance [15, 209]. The simultaneous inhibition of both partners in a HER dimer, using covalent binders that confer irreversible inhibition, represents one of these new paradigms. In this light, we will discuss two multitargeted compounds, being MEHD7945A (duligotuzumab) and afatinib.

For MEHD7945A (duligotuzumab), a monoclonal antibody with dual EGFR/HER3 specificity, we demonstrated that this compound has only a limited potential to establish a clear concentration-dependent cytotoxic effect in intrinsically and acquired cetuximab resistant HNSCC cell lines [210]. An additive but not synergistic interaction between MEHD7945A and cisplatin was observed. As the cytotoxic effect of MEHD7945A was not dependent on the expression of EGFR and HER3 in

HNSCC cell lines, other mechanisms besides HER3 expression and signalling seem to play a pivotal role in resistance to cetuximab. This finding was supported by clinical data from the MEHGAN study, a randomized phase II study comparing MEHD7945A with cetuximab in platinum-pretreated but cetuximab-naïve HNSCC patients. This study demonstrated no benefit for MEHD7945A over cetuximab in neither all randomized patients, nor in patients whose tumours expressed high levels of HER3 or neuregulin (NRG1), a ligand of HER3. In addition, MEHD7945A also demonstrated disappointing results in a clinical study with *RAS* wild type CRC patients [211]. In this study, MEHD7945A plus FOLFIRI (leucovorin, 5-fluorouracil and irinotecan) did not appear to improve the outcomes of *RAS* wild type CRC patients compared with cetuximab plus FOLFIRI. Similarly, no association was found between progression free survival or objective response rate and HER3 or NRG1 expression. Due to the lack of survival benefit reported in several clinical trials, no additional clinical studies have recently been initiated with MEHD7945A (ClinicalTrials.gov), indicating the need to further investigate the potential of other multiple HER receptor inhibitors, such as for example afatinib.

In contrast to the first-generation EGFR inhibitors, afatinib is an irreversible HER family blocker that inhibits the enzymatic activity of EGFR, HER2 and HER4 [212–215]. As HER3 is kinase-inactive and requires obligate heterodimerization with other HER-family receptors, afatinib also inhibits HER3-mediated signal transduction. The increased inhibition scope of HER receptors by afatinib most likely leads to a more robust blockade of the HER network [216]. Previous preclinical research demonstrated effective cytotoxic activity of afatinib in HNSCC cell lines and xenograft models [217]. Consequently, treatment with afatinib might result in a distinct and more pronounced therapeutic benefit.

In this light, we demonstrated that afatinib was able to establish cytotoxicity in cetuximab sensitive, intrinsically and acquired resistant HNSCC cell lines, independent of the HPV status [218]. Neither cetuximab resistance nor HPV status had a significant impact on the efficacy of afatinib. Nevertheless, we noticed that intrinsically and acquired cetuximab resistant HNSCC cell lines tended to show higher IC_{50} values compared to their isogenic cetuximab sensitive counterparts, thus suggesting the possibility of cross-resistance between cetuximab and afatinib.

In HNSCC patients, the randomized phase II study of afatinib monotherapy versus cetuximab in R/M HNSCC patients reported that afatinib showed antitumor activity comparable to cetuximab with lack of cross-resistance [219]. In contrast, however, subgroup analysis of the phase III LUX-Head and Neck 1 trial with R/M HNSCC patients progressing on or after platinum-based therapy, suggested in 2017 that afatinib is more effective in patients whose tumours are cetuximab naïve [220, 221]. Nevertheless, a phase Ib study in patients with non-small cell lung cancer and HNSCC demonstrated promising results when afatinib was given in combination with standard-dose cetuximab [222].

The above-mentioned subgroup analysis of the LUX-Head and Neck 1 trial also suggested, based on prespecified biomarker assessment, increased benefit in patients whose tumours were, HPV-negative, had EGFR amplification, low HER3 expression and high PTEN expression [221]. However, our preclinical data suggested that

the efficacy of afatinib was not significantly influenced by the HPV status of the cell line. Furthermore, in 2018, Machiels et al. reported that none of these biomarkers were significantly predictive of response for afatinib in a window of opportunity study of the European Organization for Research and Treatment of Cancer (EORTC) study in treatment-naïve HNSCC patients selected for primary curative surgery [223]. Possible explanations for these differences include the low number of patients resulting in low statistical power and the different clinical settings (curative versus palliative). Although these data were exploratory, Machiels et al. reported that the hypoxic gene signature and TP53 status needed to be further investigated as a predictive biomarker of afatinib activity. Our preclinical data support this finding, as the cytotoxic effect of afatinib was increased under hypoxic conditions in HNSCC cell lines. Consequently, further preclinical and clinical research are required to draw final conclusions upon the possible predictive role of cetuximab sensitivity, HPV status, hypoxia and TP53 status for the treatment with afatinib

Overall, the extended inhibition scope of HER receptors by afatinib leads to a more robust blockade of the HER network than MEHD7945A.Nevertheless, optimisation of combination treatment regimens with afatinib and conventional as well as other targeted therapies is necessary. Furthermore, identifying predictive biomarkers in order to select the patients that benefit most from these particular combination strategies is of crucial importance.

Identification of Drug Resistance Mechanisms and Predictive Biomarkers

In addition to optimising therapy strategies, optimal patient selection for anti-EGFR-based therapy remains a major challenge. As such, efforts at identifying predictive biomarkers to select HNSCC patients most likely to benefit from EGFR-targeted therapy have yet to succeed [15, 224, 225]. Unravelling the molecular pathways underlying resistance to EGFR inhibitors could have important implications, not only regarding patient selection, but also regarding the identification of novel drug targets for the treatment of HNSCC patients. In the paragraphs above, we discussed which mechanisms of cetuximab resistance are already known and which ones deserve further investigation. This enhanced knowledge will guide us to rationally design and test novel combination therapies that overcome resistance to EGFR-targeting agents in cancer treatment.

Conclusion and Future Perspectives

In conclusion, we hypothesize that the anti-tumour effects of cetuximab will be synergistic with agents targeting oncogenic bypass pathways responsible for therapeutic resistance towards cetuximab in HNSCC. Of particular interest and complexity are regimens combining immunotherapy with EGFR-targeted therapy. Indeed, the integration of immunotherapeutic approaches is now considered as a new perspective for the treatment of HNSCC patients. In this regard, the anti-PD-1 immune checkpoint inhibitor pembrolizumab has recently been approved by the American FDA and the European EMA for first-line treatment of R/M HNSCC [226]. As discussed previously, the working mechanism of cetuximab has largely been attributed to the direct effects of EGFR inhibition, but cetuximab also demonstrates additional immune-based mechanisms of activity through stimulation of antibody-dependent cellular cytotoxicity and enhancement of cytotoxic T-lymphocyte cross priming by dendritic cells [227–229]. As such, the immune system of the patient is involved in the anti-tumour effect of cetuximab and combinations with immunotherapeutic approaches also look highly promising for the treatment of HNSCC. We are hopeful that, with these novel combination strategies, cetuximab resistance can be prevented and a more pronounced therapeutic benefit can be achieved, ultimately resulting in improved survival and quality of life for HNSCC patients.

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