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Investigational EGFR-targeted therapies in HNSCC

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Abstract

Importance of the Field—The epidermal growth factor receptor (EGFR) is an established therapeutic target in head and neck squamous cell carcinoma (HNSCC). The EGFR-targeting monoclonal antibody cetuximab (™Erbitux) was FDA-approved for use in HNSCC in 2006. The molecular basis for the efficacy of an antibody approach compared with inhibition of EGFR tyrosine kinase function using small molecule inhibitors, or downregulation of protein expression via antisense strategies remains incompletely understood.

Areas covered in this review—A literature search was performed to identify studies elucidating mechanisms of action of several approaches to targeting EGFR in HNSCC (monoclonal antibodies, tyrosine kinase inhibitors, antisense approaches, and ligand toxin conjugates).

What the reader will gain—Monoclonal antibodies decrease tumor growth via receptor endocytosis and recruitment of host immune defenses. Tyrosine kinase inhibitors bind to the ATP binding pocket of the tyrosine kinase domain, inhibiting signaling. Antisense approaches decrease EGFR expression with high specificity although drug delivery remains problematic. Ligand-toxin conjugates facilitate the entry of toxin and the ADP-ribosylation of the ribosome, thereby inhibiting translation.

Take home message—Elucidation mechanisms by which these different strategies inhibit EGFR function may enhance the development of more effective treatments for HNSCC and enable prospective identification of individuals who will benefit from EGFR inhibition.

Keywords

EGFR; TKI; monoclonal antibodies; antisense oligonucleotides

1. INTRODUCTION

Head and Neck squamous cell carcinoma (HNSCC) is the sixth most prevalent cancer worldwide and is responsible for 90% of the cancers that arise in the mucosal surfaces of the head and neck [1]. There are 40,000 new HNSCC cases and 12,000 deaths per year in the United States alone [1]. In addition, there are 600,000 new HNSCC cases worldwide annually [2-3]. Risk factors for the development of HNSCC include tobacco smoking and alcohol consumption [1]. In addition, the Human Papilloma Virus (HPV) has emerged as a risk factor for HNSCC, primarily HPV-16 [4]. HPV16 seropositivity has been associated with increased risk for HSNCC [5]. Current treatment for HNSCC involves radiation or surgery in early stages of the disease [6]. Treatment for more advanced stages involves either radiation or surgery in combination with chemotherapy [7]. Despite improvements in surgical techniques,

chemotherapy agents and radiation delivery, long-term survival is relatively unchanged [8]. New therapeutic approaches, including molecular targeted therapies are needed to increase long-term survival.

Epidermal Growth Factor Receptor (EGFR) has been implicated in the pathogenesis of HNSCC. EGFR is overexpressed in up to 90 % of head and neck cancers compared with levels in normal mucosa [9-10] where expression levels correlate with decreased survival, independent of therapy [11-12]. Targeting EGFR using a variety of strategies abrogates tumor growth in preclinical HNSCC models [13-15]. Despite ubiquitous EGFR expression in HNSCC, only a subset of patients will respond to EGFR inhibitors, including cetuximab. Even in the setting of upstream EGFR inhibition, alternative downstream signaling pathways are persistently activated [16]. This persistent activation has been shown to result, at least in part, from signaling through other cell-surface receptors including G-protein-coupled receptors (GPCRs), Insulin Growth Factor Receptor (IGF-R) and Met [17]. Combined targeted therapy of both EGFR and key intermediates in GPCR-EGFR crosstalk pathways showed an enhanced effect on migration and invasion compared to either therapy alone [17]. New treatments must be devised to improve the low cure rate with conventional therapies and the appearance of secondary tumors after initial intervention. Knowledge of the EGFR protein and its downstream signaling effects has advanced the field of targeted agents significantly. The promise that combined therapy holds, especially in the context of combined EGFR/GPCR targeting or radiation and targeted therapy may reveal that clinical therapy may involve many of these treatment modalities. In this review, we will describe the efficacy of individual EGFR targeting strategies in the context of HNSCC. We will also discuss how the mechanisms of anti-tumor effects may confer sensitivity or resistance to specific therapeutic approaches [18].

2. HNSCC AND EGFR BIOLOGY

EGFR (erbB1, HER1), a 170 kDa cell-surface protein, regulates the growth and differentiation of cells. EGFR is a member of the erbB family of receptor tyrosine kinases, four closely related cell membrane receptors: EGFR (HER1 or erbB1), erbB2 (HER2), erbB3 (HER3), and erbB4 (HER4), which contribute to proliferation and invasion of cancer cells, including HNSCC cells, in response to stimulation with growth factors [19]. EGFR is activated in response to specific ligands, including EGFR, TGF-alpha, HB-EGF, and amphiregulin [20].

Under normal circumstances, EGF ligand binds to the extracellular domain of the EGFR and induces a conformational change. The binding of EGF to the extracellular domain (also known as domains I and III) causes a single EGF receptor to undergo a more extended conformation, due to the severing of intramolecular bonds acting upon domains II and IV [21]. In this process, domain II, a cystine-rich domain becomes exposed and is the driving force for the formation of a “dimerization arm” between two EGF-receptors [21]. These conformational changes result in the homodimerization or heterodimerization of the EGFR with other members of the ErbB family. Once dimerization occurs, the receptor becomes activated [21]. X-ray crystallography studies have shown that disrupting domains II and IV through deletion or mutation or artificially creating an extended EGFR conformation does not create an active EGFR protein. In fact, it is hypothesized that the activated form of EGFR is induced through a combination of effects, including the unfolding of EGFR and the exposure of the wild-type form of domain II [21]. This activation sequence is in contrast to other members of the receptor tyrosine kinases, which are activated by receptor-mediated dimerization instead of undergoing a stabilization upon ligand binding before activation [22].

The mechanisms by which EGFR activation occurs has implications for potential strategies to downmodulate EGFR. Therapeutic antibodies that compete for EGFR ligand binding to the

extracellular domain of the receptor, stabilize the tethered conformation, block the extended state, and directly inhibit ligand binding and receptor dimerization [23]. X-ray crystallography studies demonstrate that cetuximab competes with EGF ligand(s) by binding to an epitope on domain III of the EGFR, which significantly overlaps with the extracellular binding domain [23-24]. This binding also causes a steric blockade of the unfolding of EGFR into the extended conformation that is required for dimerization [5-6]. Direct inhibition of the dimerization domain by monoclonal antibodies to the ErbB2 form of domain II also represents a therapeutic mechanism for monoclonal antibodies. Pertuzumab blocks the heterodimerization of ErbB2 and ErbB3 with EGFR and is used for breast cancer therapy [5].

The physical juxtaposition of the receptors due to domain II has implications for EGFR activation mechanisms. Studies by Zhang and colleagues showed that the CDK/cyclin-like interface in the EGFR protein is required for receptor activation [25]. Point mutations in this interface result in abrogation of EGFR activation [25]. Studies have also focused on defining which phosphotyrosine residues are responsible for ErbB downstream signaling and what interacting partners are associated with these residues. Using a novel, high throughput screen to examine binding partners of the 89 cytosolic tyrosine residues, Matthias Mann and colleagues have determined that the binding partners of ErbB1-ErbB4 phosphotyrosine residues are conserved, such as Grb and Src [26]. Interactors of phosphotyrosine residues then function as intermediates with the Ras-Raf-Mek-MAPK, Jak-Stat, PLC-gamma, and/or the PI3K/Akt signaling pathways. These contribute to cell survival, proliferation, invasion, and metastasis [9,19].

3.1. MECHANISMS OF RESISTANCE

EGFR transactivation by GPCRs as been implicated as a mechanism of persistent downstream EGFR signaling [27] and represents one potential mechanism of resistance (Figure 1). In cetuximab resistant clones derived by prolonged *in vitro* exposure, an increase in EGFR, ErbB2 and ErbB3 was detected compared to parental lines [28]. To determine the effect of EGFR phosphorylation on the activation of Her2 and Her3, Harari and colleagues used TKIs to inhibit the 1173 phosphotyrosine residue on EGFR and examined expression levels of Her2, Her3, cMet, Akt, and MAPK [28]. Levels of these proteins were decreased compared to non-treated controls, indicating that EGFR activation contributes upregulation of Her2 and Her3, increased downstream signaling, and consequent resistance to antibodies [28]. Evidence supporting the contribution of Her2 and Her3 to cetuximab resistance involved the use of 2C4, an inhibitor to Her2 dimerization. Suppression of Akt and Her3 were seen upon treatment with cetuximab and 2C4 compared to cetuximab alone, revealing the dependence of resistant cells on Her2 expression [28]. In addition, loss of Her3 resensitizes resistant cell lines to cetuximab, implicating Her3 in resistance [28].

In addition to increased transactivation of EGFR with Her2 and Her3 conferring resistance to therapy, genomic amplification can also result in resistance. EGFR copy number was assessed through the ratio of the real-time PCR level of EGFR vs. Met in ten HNSCC lines. Twenty percent of the cell lines showed relative copy numbers greater than 5 and half of the cell lines tested revealed a copy number between 2 and 5, indicating a low to moderate amount of EGFR amplification [14]. In addition, high EGFR copy numbers was statistically associated with cetuximab and gefitinib resistance [14]. High expression of ErbB2 and ErbB3 has also been implicated in gefitinib resistance where increased levels or ErbB2 and ErbB3 expression correlated with high IC50s in three HNSCC cell lines [14]. Other studies have shown that EGFR FISH (Flourescent *in situ* hybridization) copy number has been implicated in poor prognosis [29]. Chung and colleagues have found that in 75 HNSCC tumor samples, 58% were FISH positive and that tumor differentiation was weakly associated with FISH status [29].

FISH status was also a significant prognostic indicator of progression-free and overall survival [29].

Kinase domain mutations in of EGFR in HNSCC are extremely rare but may be associated with altered responses to EGFR inhibitors when they occur [30]. In one study, tumor samples of 100 patients with advanced primary or relapsed HNSCC were analyzed by PCR. Results revealed that one patient K745R mutation in the ATP binding site. This mutation may confer resistance to TKIs due to the stabilization of residues involved in binding to both ATP and TKIs [30]. This mechanism has been hypothesized to explain TKI resistance in NSCLC, involving a mutation in close proximity to the K745R mutation.

In addition, the induction of the epithelial to mesenchymal transition (EMT) has been shown to be a marker of resistance to EGFR-targeted therapy [31]. In high risk HNSCC, loss of tight and adherens junctions, dysregulation of E-cadherin and the conversion of cells to a more spindle-shaped morphology facilitates movement across the basement membrane and increased metastasis [32]. The resistance to tyrosine kinase inhibitors may be due to the expression of proteins like vimentin and loss of the epithelial cell adhesion molecule EpCAM [31]. In addition, the phenotype of the cell itself rather than the expression of proteins may be responsible for TKI resistance. The loss of cell to cell adhesion through decreased claudin expression and increased cell motility confer a more aggressive phenotype to the tumor and may explain resistance [31]. These changes in gene expression has been shown to predict gefinitib resistance in lung cancer and HNSCC models [31].

Furthermore, EGFR transactivation by GPCRs has been implicated as another mechanism of persistent downstream signaling and resistance [27]. When used as monotherapy, EGFR inhibitors show little success in treating HNSCC and suggest a collateral mechanism may be involved. Upon stimulation of GPCRs, the release of EFGR ligand increases, explaining continued EGFR signaling. In addition, GPCRs have also been shown to act in both EGFR-dependent and independent mechanisms. Bradykinin (BK) and lysophosphatidic acid (LPA), two GPCR ligands, have been shown to activate MAPK in both the presence and absence of inhibitors to EGFR [27]. Interestingly, the EGFRvIII variant requires no ligand binding for activation and represents another mechanism of resistance. The EGFRvIII variant results from a deletion of exons 2 through 7, which codes for the extracellular binding domain [33]. The resulting protein is 150kDa and is weakly constitutively active; the absence of a binding domain precludes binding of monoclonal antibodies to the protein. EGFRvIII transfected cells have been shown to be more resistant to cetuximab when compared to vector-transfected cells *in vitro* and *in vivo* [33]. Activated EGFR may then activate pathways that are responsible for resistance, including STAT proteins, and the PI3K/Akt pathway.

3.2 Preclinical Data and Mechanisms

Monoclonal antibodies exert their effects by binding to the extracellular domain of the EGFR and competing for ligands including TGF-alpha and amphiregulin, which act as autocrine ligands for EGFR [34-35]. Unlike endogenous ligands, which stimulate downstream EGFR effectors, monoclonal antibodies result in the abrogation of signal transduction [36]. Cetuximab abrogated EGFR-induced upregulation of EGFR, determined by decreases in Erk1/2 activation [36]. In contrast, when these cells were incubated with EGF and cisplatin alone, there was an upregulation of EGFR [36]. This suggests that abrogation of EGFR activation is mediated by competitive binding of the antibody to its receptor.

In addition to competitive binding, increased degradation and internalization of the EGFR has also been shown to be a mechanism by which monoclonal antibodies decrease EGFR signaling [37]. In an effort to investigate the cellular localization of the EGFR in the presence of monoclonal antibodies, HNSCC cells treated with EGFR monoclonal antibodies showed a

decrease in the cell surface expression of EGFR compared to controls [37]. Internalization of the receptor prevents EGFR from binding to its ligands and thereby interferes with signaling.

X-ray crystallography studies performed by the Ferguson lab have shown that the humanized antibody Fab11F8 interacts exclusively with domain III, preventing EGF from binding to domain III and results in the tethered phenotype [24]. In addition, most of the interactions of Fab11F8 involve interactions between domain III and the heavy-chain complementary-determining regions (CDRs) [24]. H1 lies at the center of the interaction between V_H and domain III and stabilizes the interaction between H2 and H3 [24].

The structural basis for antibody targeting is not the only mechanism that makes antibodies effective at downmodulating EGFR/ErbB expression, however. Cross-linking and surface depletion has been shown to be instrumental as well. The Yarden lab has shown that antibodies engages endocytosis of EGFR at a much slower rate than EGF, as determined by biotinylation and Western blot [38]. Combinations of antibodies directed[39] at different epitopes of EGFR caused a synergistic downregulation of EGFR [38]. This downregulation, then, seems to be dependent on the amount of cross-linking at the cell surface [38].

Monoclonal antibodies may also exert their anti-tumor properties via their effects on the immune systems of patients with HNSCC. Patients with HNSCC may exhibit upregulation of angiogenic and inflammatory factors that promote tumorigenesis, like VEGF[40]. Much work has been performed to evaluate the effects of using antibodies to neutralize the effects of VEGF in a variety of tumors. Interestingly, one side effect and challenge of therapy against VEGF is a compensatory increase in circulating VEGF levels [40-41]. Other factors include increased production of proangiogenic factors including IL8 and a dysregulation in the cytotoxic ability of effector T-cells [42-43]. Antibodies have demonstrated the ability to increase the cytotoxic capacity of natural killer cells and dendritic cells in head and neck cancer cells *in vitro* [42-43]. In addition, monoclonal antibodies also competitively binding to the EGFR, increase internalization and degradation of the EGFR, and induce antibody-dependent cell-mediated cytotoxicity (ADCC).

Chimeric monoclonal antibodies, by virtue of their Fc constant regions, can elicit ADCC, which may contribute to the clinical response mediated by monoclonal antibodies by recruiting effector cells [4-5]. In the process of ADCC, cells of the innate immune response bind to the constant regions of antibodies through their Fc-gamma receptors. Opsonization of a cancer cell with antibodies to EGFR with consequent binding of effector cells to these antibodies results in phagocytosis and cell lysis [39]. Both cetuximab and panitumumab have been shown to induce ADCC in the HNSCC cell lines. Cytotoxicity assays showed that in the presence of peripheral blood mononuclear cells and either cetuximab or panitumumab, levels of lactate dehydrogenase were significantly higher than compared to controls, indicating greater levels of cell lysis [39]. This finding suggests that ADCC represents a possible mechanism by which monoclonal antibodies exert their antitumor activity. The Fc gammaR constant regions on effector cells also contribute to the mechanism of *in vivo* cytotoxicity. Mice deficient for Fc gamma receptor IIB maintained a protective effect while mice deficient in Fc gamma receptor III showed an increase in tumor activity upon treatment with monoclonal antibodies [44]. In addition, antibody efficacy was enhanced by polymorphisms in the Fc receptor region, in the setting of rituximab treatment for lymphoma [45]. The Fc gamma RIIIa 158 valine/valine and the Fc gamma RIIa 131 histidine/histidine polymorphisms were independently associated with outcome [45]. In addition, more polymorphisms that seemed to bestow greater response in patients with metastatic colorectal cancer are the Fc gamma IIIa V/F and IIa V/R polymorphisms [27]. Other mechanisms involved in the efficacy of monoclonal antibodies comes from examining the effect of inhibiting human adenocarcinoma cell lines with C225. Work performed by the Mendelsohn lab show that G1 cell cycle arrest is induced upon

administration of C225 to DiFi cells. This cell cycle arrest is also dependent on p27Kip1 [46].

4. MONOCLONAL ANTIBODIES

4.1. Clinical Data

Monoclonal antibodies represent a substantial subset of current targeted therapy toward EGFR and have exhibited clinical benefit. Cetuximab (C225, erbitux), a chimeric, mouse-human IgG1 monoclonal antibody, was approved by the FDA in 2006 in combination with radiation for the treatment of HNSCC. More recently, cetuximab plus chemotherapy was shown to be effective in recurrent/metastatic HNSCC [47]. Patients were randomized to receive either platinum plus fluorouracil or platinum, fluorouracil, and cetuximab. Median progression-free survival was 5.6 months in the treatment group vs. 3.3 months in the group that did not receive cetuximab. Based on promising results in two phase I and II trials [48-49], the clinical development of, cetuximab, was rapidly tested in three phase III trials [47,50-51]. While phase III studies demonstrated improved clinical responses when cetuximab was added to radiation [51] or chemotherapy [47,50], the combination of radiation and cetuximab significantly enhanced HNSCC survival. This led to rapid FDA approval of cetuximab for the treatment of newly diagnosed HNSCC [50-51]. A definitive randomized phase III clinical trial is underway to investigate cetuximab plus chemoradiation therapy (RTOG 0522).

Ongoing studies are testing the benefit of adding cetuximab to concurrent chemoradiation therapy (CRT) compared to chemoradiation alone, due to the promise of combined cetuximab and chemotherapy [51-52]. In addition, trials are evaluating the benefit of administering cetuximab prior radiation therapy as a radiosensitizing approach [53]. Panitumumab, a fully humanized IgG2 monoclonal antibody, is being evaluated in phase II clinical trials to determine its clinical benefit, as it has shown much promise in metastastic colorectal cancer (ClinicalTrials.gov NCT00547157). Zalitumumab and nimotuzumab are also being investigated in the context of combination therapies for HNSCC, as these may have enhanced ADCC properties (Zalitumumab) and unique binding sites (nimotuzumab) (Table 1).

Despite ubiquitous EGFR expression in HNSCC tumors, only a subset of patients will respond to EGFR-directed antibody therapy. Elucidation of potential mechanisms of response and/or resistance may facilitate more effective use of these therapies. EGFR expression has not generally served as a marker of clinical response. One Phase III clinical trial found that the combination of cetuximab plus cisplatin improved response rates when compared to cisplatin alone in HNSCC. Clinical responses were greater for patients with EGFR staining in less than 80% of their tumor cells [50]. Harari and colleagues generated a cetuximab-resistant HNSCC cell line (UM-SCC1) *in vitro* and found that these cells expressed higher levels of Her2 and Her3 as well as increased hereodimerization of Her2 or Her3 with EGFR [28]. Downmodulating the expression of these Her family members with small molecules restored cetuximab sensitivity [28]. Trials of pan-HER inhibitors are underway in HNSCC.

The EGFRvIII variant contains a truncated ligand-binding domain, which may influence its binding to monoclonal antibodies [54]. EGFRvIII expressing tumors have shown decreased *in vitro* response to cetuximab [33]. EGFRvIII expression is therefore a plausible mechanism of cetuximab resistance. In an EGFRvIII model, xenografts were resistant to cetuximab treatment [33]. Additional models of EGFR antibody resistance are needed that mimic the clinical scenario to more fully elucidate mechanisms of acquired and de novo resistance in HNSCC. While the structure of EGFR may present some challenges for targeted therapy, a novel monoclonal antibody, mAb806, has been raised that is active against wild type EGFR as well as the EGFRvIII variant [55]. mAb806 recognizes an epitope that is present in cells that overexpress wild type EGFR and EGFRvIII [55]. Murine mouse models show that

mAb806 results in dramatic tumor regression in lung cancer models compared with cetuximab [55]

5. TYROSINE KINASE INHIBITORS

5.1. Preclinical Data and Mechanisms

Tyrosine kinase inhbitors (TKIs) cause an abrogation of EGFR signaling through preventing the phosphorylation events necessary for downstream signaling. When ligand is bound to EGFR, the intracellular domain unfolds. Domain II is exposed and forms a bridge with the domain II of another member of the ErbB family. This dimerization results in the transphosphorylation of tyrosine residues in the activation loop (A-loop) in the C-terminal portion of EGFR [56]. There is also conformational change in the A-loop that reduces the steric hindrance associated with ligand binding. Two important interactions take place. There is the interaction between Tyr845 and Glu848, important for activation loop conformation [56]. In addition, there is a hydrogen-bond interaction between Tyr845 and Arg812, which is critical for the catalytic machinery responsible for transfer of a phosphate [56]. Furthermore, there is a three amino acid sequence, Leu-Val-Ile (LVI) sequence near the carboxy-terminus that is hypothesized to regulate transphosphorylation events [56]. After LVI sequence regulation, the catalytic domains on either side of the kinase domain (N and C lobes) interact [56]. This interaction is dependent on binding of ATP in the ATP cleft located between the N and C lobes at K745 [56-57]. After initial tyrosine phosphorylation, there is subsequent phosphorylation of docking sites [56]. Adaptor proteins that contain an SH2 domain can then associate with phosphotyrosine residues located on the carboxy-terminal portion of EGFR on these docking sites. Tyrosine kinase inhibitors like gefitinib and erlotinib are anilinoquinazolines which bind reversibly to the K745 site in the ATP binding pocket [57].

Mutations in the tyrosine kinase domain of EGFR are rare in patients with HSNCC (1% of Caucasian and 7% of Asian individuals) [33]. However, tyrosine kinase mutations are frequently seen in NSCLC and have offered much insight into the structure and function of EGFR. One common mutation seen in NSCLC patients that bestows sensitivity to TKIs is the L858R mutation [58-59]. This mutation is a gain of function mutation and also located in a group of amino acids near the ATP binding cleft known to regulate binging of TKIs [59]. It is hypothesized that the disruption of these critical residues results in the stabilization of their interaction with ATP and with gefitinib [59]. Retrospective studies have been performed to evaluate tumor response to gefitinib or erlotinib in the context of the L858R and exon 19 mutations (which also has been shown to confer sensitivity) [60]. Results show that clinical response was better and time to progression was longer for those patients that harbored the exon 19 deletion [60]. One possible explanation is that exon 19 deletions result in greater inhibition by gefitinib and erlotinib, but such conclusions are not supported by *in vitro* data [60]. Most of the activating mutations have been shown to be in exons 18-21 in NSCLC [61].

Other mutations, however, result in resistance to tyrosine kinase inhibitors. The T790M mutation is a common mutation seen in gefitinib and erlotinib resistant NSCLC; this mutation has been shown to change the conformation of the ATP binding site such that these drugs have an extremely low binding affinity [58]. The D761Y mutation,, is located in the alpha-C-helix of EGFR adjacent to E762 [58]. E762 forms a salt bridge with L745 that interacts with ATP and may explain why the D761Y mutation confers the resistant phenotype [58].

Using *in vitro* models of HNSCC, the decrease in activation of EGFR through tyrosine kinase inhibitors results in decreased growth. The proliferation of HNSCC cell lines is reduced following treatement of the preclinical EGFR-specific tyrosine kinase inhibitor PD153035 [13]. In addition to decreased growth, tyrosine kinase inhibitors abrogate the activation of downstream effectors of EGFR, including Akt and Erk, which serve as markers of cell survival

and proliferation [62]. Downmodulation of EGFR kinase activity through erlotinib-treated cell lines also leads to the induction of apoptosis [1]. In tumors harvested from mouse xenograft models, gefitinib was shown to decrease markers of proliferation, such as Akt, Erk, and Ki67 [63].

Preclinical HNSCC animal models demonstrated a modest decrease in tumor volume after 40 days of EGFR TKI treatment when compared to control [64]. Combination treatment using gefitinib and IFN-alpha (an antitumor cytokine) resulted in marked decreases in tumor volume when compared to gefitinib or IFN-alpha alone or polyethylene glycol treated control groups. Other studies using the EGFR-specific tyrosine kinase inhibitor CP-358,774 reported significant reduction in tumor volume when compared to controls [65].

5.2. Clinical Data

Clinical trials to date have reported modest benefits with the use of EGFR tyrosine kinase inhibitors in HNSCC. A multicenter phase II trial to evaluate the effects of erlotinib on recurrent/metastatic oral squamous cell carcinoma showed an overall response rate of 4.3% and disease stabilization for a median of 16.1 weeks in 38.3% of patients [66]. Median overall survival was 6.0 months [66]. Other phase II clinical trials have showed similar responses using single agent therapy [67]. A phase II study examining the combination of radiotherapy and gefitinib in HNSCC reported 32% complete remission and 53% partial remission [68]. However a phase III trial demonstrated no survival advantage when gefitnib (Iressa) was combined with methotrexate for the treatment of recurrent/metastatic HNSCC [69]. While the clinical benefit of EGFR TKI in HNSCC is met with limited success, to date ongoing studies will determine the role of these agents in HNSCC. For example, clinical trials are evaluating combination studies using combination tyrosine kinase inhibitor and monoclonal antibody in HNSCC (Table 2).

5.3. Monoclonal Antibodies vs. Tyrosine Kinase Inhibitors

Several mechanisms may explain the differences in clinical efficacy of EGFR monoclonal antibodies compared with tyrosine kinase inhibitors. Tyrosine kinase inhibitors do not induce ADCC or internalization of receptor. As a result, the immune system of the host is not harnessed and there is no decrease in surface expression of EGFR. In addition, monoclonal antibodies may demonstrate greater efficacy in HNSCC may be due to the lack of tyrosine kinase mutations in this cancer [33]. Tyrosine kinase inhibitors have been shown to be very effective in decreasing tumor burden in non-small cell lung carcinoma (NSCLC) in patients who harbor mutations in the tyrosine kinase domain of EGFR [70].These mutations allow for greater sensitivity and increased response to tyrosine kinase inhibitors [70]. However, tyrosine kinase mutations of EGFR in HNSCC are exceedingly rare [33]. As a result, one would not expect the increased sensitivity to tyrosine kinase inhibitors in HNSCC such as that seen in NSCLC. In addition, many HNSCC cell lines demonstrate IC_{50} values above the 10 micromolar range [71]. This suggests that these cell lines are relatively resistant to tyrosine kinase inhibitors. The dose required to cause downregulation of EGFR through tyrosine kinase inhibitors in patients may not be practical.

6. ANTISENSE OLIGONUCLEOTIDES AND siRNA

6.1. Preclinical studies and mechanism

Antisense oligonucleotides against EGFR are in the early stage of clinical investigation based on promising preclinical findings. Antisense oligonucleotides consist of a stretch of a single stranded DNA molecule of ~20 nucleotides in length. They exert their anti-tumor effect through binding of mRNA, sterically hindering ribosomes and preventing translation [72]. Unmodified antisense oligonucleotides are susceptible to degradation by endonucleases and exonucleases.

Chemical modification of oligonucleotides with phosphorothioate moieties enhances resistance to nuclease degradation [73]. Cloning of the antisense oligonucleotide into a gene expression construct allows for the delivery of increased concentrations of the antisense sequence into the tumor. Systemic delivery however, is relatively challenging when compared to other modes of targeted agents (Table 3).

The ability of antisense therapy to reduce translation is illustrated by preclinical studies downregulating EGFR in HNSCC. Xenograft studies demonstrated that tumors treated with EGFR antisense gene therapy were significantly smaller than vector treated controls [74]. Furthermore, EFGR-antisense treated tumors demonstrated decreased EGFR expression and increased apoptosis through staining for DNA fragmentation [74]. An antisense strategy has also been used to target the EGFR ligand, TGF-alpha [75]. HNSCC xenografts treated with TGF-alpha antisense DNA plus liposomes showed an inhibition of tumor growth when compared to controls [75]. Moreover, this antitumor effect was further supported by decreases in Bcl-X_L, an apoptotic protein [75]. The mechanisms of antisense oligonucleotides and TGF-alpha-specific antibodies may be similar [75]. DNA synthesis decreases upon treatment of HNSCC and ovarian cancer cell lines with TGF-alpha antibodies [75].

EGFR antisense approaches may be enhanced by combinations with chemotherapy. Targeting EGFR protein production with antisense oligonucleotides and docetaxel in a HNSCC xenograft model resulted in downmodulation of EGFR, Akt, and Ki67 expression, as well as significant decreases in tumor volume when compared to those mice treated with docetaxel alone [76]. The increased response of this combined approach may result from the use of several methods to target EGFR, as docetaxel has also been shown to decrease EGFR expression [40-41]. While more conventional strategies to inhibit EGFR including antibodies and kinase inhibitors result in a response in about 5-10% of patients, their limited efficacy might stem from receptor turnover and a lack of full blockade of the receptor [76]. The addition of antisense approaches may be a way to circumvent these barriers to treatment.

siRNA strategeies are emerging as potential approaches to target gene expression. siRNA consists of double-stranded RNA, containing a sequence necessary to silence the translation of a target protein [77]. When the stand of RNA enters cells, it is fragmented by the enzyme Dicer, which creates 20 bp double stranded RNA molecules with 2-nucleotide 3′ overhangs [78]. These strands of RNA bind to the RNA-induced silencing complex (RISC), unwind, and then are guided by RISC to its complementary mRNA strand [79]. The RISC complex contains slicer, which cleaves target mRNA [80]. Preclinical studies using siRNA have shown that it can significantly decrease EGFR expression compared to scrambled siRNA-transfected controls, as evaluated by flow cytometry [15]. In addition, HNSCC xenografts treated with EGFR siRNA and cisplatin revealed a significant decrease in tumor burden when compared to controls [15].

While preclinical studies using siRNA to downregulate EGFR expression in HNSCC are limited, others have used this strategy in glioblastoma cell lines and xenograft mouse models. Glioblastoma, like HNSCC, has been shown to be associated with overexpression of EGFR [81]. EGFR siRNA-transfected glioma cells proliferated less than vector-transfected controls [82]. Furthermore, glioma xenografts treated with EGFR siRNA demonstrated a significant reduction in tumor volume ($p<0.01$) when compared to controls [82]. One interesting advantage that siRNA may have over conventional EGFR targeted therapies is that siRNA can be designed to specifically decrease EGFRvIII expression [82]. Several studies using siRNA engineered to the Exon1/Exon8 junction, reported decreased Akt levels and cell cycle arrest [82-83].

6.2. Clinical studies

While early phase clinical trials of antisense oligonucleotide therapy against Bcl-2 demonstrated that it was well tolerated and able to downregulate the expression of Bcl-2, no significant therapeutic benefit was demonstrated in phase III studies and this treatment failed to receive FDA approval [84]. We have shown that in a Phase I study, antisense oligonucleotides, when delivered using an expression construct, are well-tolerated and exhibit antitumor activity in patients. In a cohort of twenty patients, no grade 3 or 4 toxicities were reported. In addition, median survival was 5.4 months [85]. The disease-control group, as defined by complete remission, partial remission, or stable disease, demonstrated a median survival of 7.9 months as compared to 3.4 months for the partial disease group [85]. Another clinical trial is currently in progress investigating antisense oligonucleotide therapy in combination with radiation and cetuximab in HNSCC (ClinicalTrials.gov NCT00903461). A phase II trial will sonn open to investigate the effects of adding EGFR antisense gene therapy to cetuximab plus radiation for advanced locoregional HNSCC. (ClinicalTrials.gov NCT00903461)

Phase I studies are also being conducted using siRNA in solid tumors, directed towards the M2 subunit of ribonucleotide reductase, a well-known cancer target. SiRNA is also being investigated in a Phase I study for metastatic melanoma, targeting the immunoproteasome subunits LMP2, LMP7, and MECL1 (ClinicalTrials.gov NCT00672542. However, clinical studies are yet to be performed using siRNA to target EGFR in the context of HNSCC.

7. LIGAND AND ANTIBODY-TOXIN CONJUGATES

7.1. Preclinical Studies

Other strategies involve targeting EGFR with conjugated toxin plus anti-EGFR antibody or a toxin conjugated to an EGFR ligand. Ligand-toxin conjugates exert their anti-tumor effects by binding to their targets leading to the internalization of the toxin. Once internalized, the toxin ADP-ribosylates proteins involved in translation [86]. Toxins, such as a modified version of the *Pseudomonas* toxin, alpha PE-38, have been conjugated to TGF-alpha and have demonstrated efficacy in a glioblastoma xeongraft model [87]. Moreover, antibodies conjugated to *Pseudomonas* toxin have shown to inhibit the growth of HNSCC *in vitro* and *in vivo*. An advantage of ligand-toxin conjugates compared with monoclonal antibodies is the variable nature of the target antibodies. Antibodies can be subject to decreased internalization to antibody-target complexes, variable expression of tumor antigens, and limited cytotoxicity [87]. TGF-alpha conjugated to alpha-PE38, was used in HNSCC models, where cleaved PARP, a marker of apoptosis, was significantly higher in HNSCC cells that received treatment as opposed to controls [88]. Mice treated with this toxin-ligand conjugate intratumorally, exhibited a decrease in tumor volume and increased numbers of tumor cells when compared to controls [88]. Other preclinical studies have investigated bivatuzumab mertansine, an antibody-toxin conjugate directed against CD44v6, a HNSCC tumor-associated cell-surface protein. In this mode of therapy, bivatuzumab, a humanized IgG1 was conjugated to mertansine, a member of the vinca alkaloids and an inhibitor of microtubule assembly [89]. In HNSCC xenografts, bivatuzumab mertansine showed dose-dependent antitumor efficacy and stable regression of tumor when compared to controls [89]. The efficacy of immunotoxins have also been shown to be effective in Her2-positive breast cancer. Conjugating trastuzumab with the microtubule-depolarizing agent maytansinoid resulted in inhibition of proliferation in trastuzumab-resistant breast cancer cell lines [90]. Furthermore, tumor regression using this approach was evident in mouse models of breast cancer [90].

7.2. Clinical studies

Ligand-toxin conjugates have demonstrated some clinical efficacy and safety in phase I trials for glioblastoma multiforme [91].The adverse effects associated with therapy were not due to the toxin itself, but to pharmacokinetic considerations including infusion volume, tumor, or catheter placement for delivery of drug [91]. Two out of fifteen patients with radiographic evidence of glioblastoma showed some response to treatment Clinical trials using this approach are few in HNSCC. One Phase I study investigating the safety of bivatuzumab mertansine in HNSCC revealed that ten percent of participants experienced an adverse event and 29 out of 32 patients experienced a serious adverse event [89]. While the study had to be terminated prematurely, preliminary investigation shows partial response in 3 patients, including the restoration of the ability to swallow [89].

8. EXPERT OPINION

EGFR is overexpressed and aberrantly activated in the majority of HNSCC tumors. To date, EGFR expression and/or gene amplification in the tumor has not been a consistent predictor of response to EGFR targeted therapies [92]. Current treatment strategies using EGFR inhibitors generally combine EGFR targeting agents with standard approaches including with chemotherapy and/or radiation. Targeting EGFR in combination with with inhibitors of other cellular signaling pathways including Src family kinases, STAT3, Met, and IGF-1R, among others, in HNSCC preclinical models, has shown promise [93-96]. Monoclonal antibodies targeting EGFR have been most extensively studied and were FDA-approved for use in HNSCC in 2006. EGFR-specific tyrosine kinase efforts are under active investivation. Current research efforts are focused on prospectively identifying those individuals who are likely to benefit from EGFR targeting strategies. Additional preclinical HNSCC models of EGFR inhibitor resistance (both de novo and acquired) are needed to further elucidate mechanisms of resistance to EGFR targeting agents and facilitate the development of more effective approaches. Most importantly, tumor biopsies from patients treated with EGFR inhibitors are required to determine both the effect of these agents on the human HNSCC tumor and determine the characteristics of tumors that fail to respond to EGFR targeting agents, despite expression of this growth factor receptor.

Article Highlights

- EGFR is upregulated in HNSCC. The ability of EGFR gene expression levels to predict response to therapy are currently being studied.
- Monoclonal antibodies, tyrosine kinase inhibitors, antisense therapy, and ligand-toxin conjugates have all shown promise in preclinical studies and are under active investigation.
- Combination therapies of targeted agents and of targeted agents with conventional chemotherapy and radiation are currently in clinical trials.
- Developing the most efficient targeted therapy modality involves increased understanding of the mechanisms involved in EGFR-mediated carcinogenesis.

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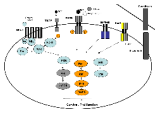

Figure 1.

Signaling pathways that may contribute to resistance to EGFR inhibitors in HNSCC. Binding of ligand to EGFR induces a conformational change that trigger molecular cascades responsible for survival and proliferation. G-protein-coupled receptors (GPCRs) maintain persistent EGFR signaling in the presence of EGFR inhibitors. The loss of E-cadherin and tight-junction expression and the transition of tumor cells from an epithelial to transitional morphology also contribute to cell survival. Her2 overexpression and consequent increased heterodimerization also results in increased downstream EGFR signaling and is associated with cetuximab resistance. The EGFRvIII variant is also associated with resistance; its truncated extracellular binding domain and constitutive signaling decreases response to cetuximab. EGFR: Epidermal Growth Factor Receptor; PI3K: Phosphoinositide 3-kinase; PDK1: Phosphoinositide-dependent kinase 1; mTOR: mammalian Target of Rapamycin; Ras: Renin-angiotensin system; Raf: Relative angiostatic factor; MAPK: Mitogen-activated protein kinase; Mek: MAPK kinase; Jak: Janus kinase; STAT: Signal Transducers and Activators of Transcription.

Table 1

EGFR monoclonal antibodies in HNSCC clinical trials

Agent	Phase	Stage	Therapy	Reference	Uniqueness of Agent/Study
Cetuximab	I	Advanced solid tumors	Cetuximab plus bortezomib (proteasome inhibitor)	[97]	Preclinical data suggests enhanced effect of combining these two agents
Cetuximab	II	Recurrent/Metastatic	Cetuximab + BIBW2992 (ErbB2 TKI)	ClinicalTrials.gov NCT00514943	Comparing C225 vs. TKI in platinum refractory patient
Cetuximab	III	Recurrent/Metastatic	Cisplatin vs. Cisplatin plus cetuximab	[50]	Prior clinical efficacy shown in combined therapy
Cetuximab	II	Resectable Stage III/IV	Cetuximab plus carboplatin, placlitaxel	[98]	
Cetuximab	II	Stage III/IV	Cetuximab plus cisplatin/docetaxel plus radiation	ClinicalTrials.gov NCT00084318	
Cetuximab	II	Stage III/IV	Cetuximab plus radiation and chemotherapy	ClinicalTrials.gov NCT00646659	
Hu-Max (Zalitumumab)	I/II	Recurrent/Metastatic	Monotherapy	ClinicalTrials.gov NCT00093041	Possible antineiplastic and ADCC mechanisms demonstrated by the antibody Zalitumumab

Agent	Phase	Stage	Therapy	Reference	Uniqueness of Agent/Study
Nimotuzumab	II	Localy Advanced	Nimotuzumab plus cisplatin/radiotherapy	ClinicalTrials.gov NCT00702481	Nimotuzumab demonstrates unique heavy chain sequences and exhibits a good safety profile
Panitumumab	I	Locally Advanced HNSCC	Panitumumab plus induction chemotherapy	[99]	Has shown promice in metastatic colorectal cancer
Cetuximab and Erlotinib	I/II	Advanced (Phase I)	Cetuximab plus erlotinib	ClinicalTrials.gov NCT00397384	
Cetuximab	III	Stage III/IV	Cetuximab plus Radiotherapy vs radiotherapy alone	[100]	

Table 2
EGFR tyrosine kinase inhibitors (TKI) in HNSCC clinical trials

Agent	Phase	Stage	Therapy	Reference
Cetuximab and Erlotinib	I/II	Advanced (Phase I)	Cetuximab plus erlotinib	ClinicalTrials.gov NCT00397384
Erlotinib	I/II	Early Stage (I/II)	Erlotinib plus celecoxib	[101]
Erlotinib	I/II	Locally Advanced HNSCC	Erlotinib plus Bevacizumab (VEGF monoclonal antibody)	[102]
Erlotinib	II	Stage III/IV	Erlotinib plus docetaxel plus Radiation therapy	[103]
Gefitinib	I	Advanced/Recurrent	Gefitinib plus paclitaxel and radiation therapy	[104]
Gefitinib/ZD1839	I	Locoregional, recurrent	Radiotherapy plus cisplatin	ClinicalTrials.gov NCT00185835
Lapatinib	I/II	Locally Advanced	Lapatinib plus combined chemotherapy and radiation therapy	[105]

Table 3
Comparision of Potential Advantages and Disadvantages of EGFR Targeting Strategies

	Advantages	Disadvantages
Monoclonal Antibodies	• High specificity for target • Induces antibody-dependent cell mediated cytotoxicity (ADCC) • Mild side effects	• Size of monoclonal antibodies may inhibit tissue penetration and clearance • IV administration
Tyrosine Kinase Inhibitors	• Oral administration • Mild side effects	• Low specificity for target, resulting in off target effects
Antisense Oligonucleotides/Gene Therapy	• High specificity for target	• Systemic administration a challenge • Delivery via cationic polymers can be toxic
Ligand-Toxin Conjugates	• Sustained activity even if patient is immunocompromised	• May be immunogenic • Hepatotoxicity common