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Protein-intrinsic and signaling network-based sources of resistance to EGFR- and ErbB family-targeted therapies in head and neck cancer

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Abstract

Agents targeting EGFR and related ErbB family proteins are valuable therapies for the treatment of many cancers. For some tumor types, including squamous cell carcinomas of the head and neck (SCCHN), antibodies targeting EGFR were the first protein-directed agents to show clinical benefit, and remain a standard component of clinical strategies for management of the disease. Nevertheless, many patients display either intrinsic or acquired resistance to these drugs; hence, major research goals are to better understand the underlying causes of resistance, and to develop new therapeutic strategies that boost the impact of EGFR/ErbB inhibitors. In this review, we first summarize current standard use of EGFR inhibitors in the context of SCCHN, and described new agents targeting EGFR currently moving through pre-clinical and clinical development. We then discuss how changes in other transmembrane receptors, including IGF1R, c-Met, and TGF- β , can confer resistance to EGFR-targeted inhibitors, and discuss new agents targeting these proteins. Moving downstream, we discuss critical EGFR-dependent effectors, including PLC- γ ; PI3K and PTEN; SHC, GRB2, and RAS and the STAT proteins, as factors in resistance to EGFR-directed inhibitors and as alternative targets of therapeutic inhibition. We summarize alternative sources of resistance among cellular changes that target EGFR itself, through regulation of ligand availability, post-translational modification of EGFR, availability of EGFR partners for heterodimerization and control of EGFR intracellular trafficking for recycling versus degradation. Finally, we discuss new strategies to identify effective therapeutic combinations involving EGFR-targeted inhibitors, in the context of new system level data becoming available for analysis of individual tumors.

Keywords

PLC- γ ; PI3K; PTEN; SHC; GRB2; RAS; STAT; IGFR; c-MET

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1. Introduction

Squamous cell cancers of the head and neck (SCCHN) encompass malignancies of the oral cavity, larynx, nasopharynx and pharynx, and are diagnosed in over 500,000 patients worldwide each year, accounting for 5% of all malignancies (Jemal et al., 2007). It is estimated that 49,260 patients develop head and neck cancer annually in the United States (Jemal et al., 2010). Treatment decisions are tailored to the primary site of disease, feasibility of organ preservation, prognosis and functional outcomes post-therapy. A multi-disciplinary team approach is often required in order to treat with curative intent.

An association between cigarette smoking and p53 mutation in head and neck cancer was reported by Sidransky in 1995 (Brennan et al., 1995). Conserved regions of the p53 gene were sequenced in tumor samples from 129 patients with squamous cell carcinoma of the head and neck. Mutations in p53 were present in 42% of the patients: 58% of those who smoked and used alcohol compared with 17% of those who neither smoked nor drank alcohol ($P = 0.001$). The hazard ratio for death was 1.4 in the face of any p53 mutation ($P = 0.009$) and 1.7 ($P < 0.001$) if a disruptive p53 mutation was identified. This remained an independent prognostic factor in multivariate analysis (Poeta et al., 2007).

Although alcohol and tobacco use has represented the likely predominant cause of SCCHN, the incidence of a second class of SCCHN related to oncogenic human papillomavirus (HPV) infection is increasing, with four-fold greater prevalence from 1984 to 2004 (Chaturvedi et al., 2011). A large Intergroup trial E4393 collected tissue from tumor and margin in patients undergoing definitive resection (Gillison et al., 2008). p53 mutations were found in 224 of 420 patients (53%). In a hospital-based, case-control study, 240 patients with SCCHN were analyzed for HPV-16 status along with patients without cancer. HPV-16 was detected in 92 case subjects and was independently associated with sexual practices and marijuana use. This was in direct contrast to the HPV-16-negative SCCHN, which was associated with tobacco and alcohol use, but not with drug use or sexual behavior. In one series, tumors from 253 newly diagnosed SCCHN patients were analyzed for the presence of the HPV genome with in situ hybridization as well as PCR based assays (Gillison et al., 2000). HPV coding regions were detected in 25% of these cases and tumorigenic HPV-16 was characterized in 90% of the HPV positive tumors. A higher tumor grade, basaloid histology, as well localization to the oropharynx were independently associated with HPV positivity (Begum and Westra, 2008) D'Souza et al., 2007). HPV-positive oropharyngeal cancers were less likely to occur among moderate to heavy smokers, and although both HPV16-positive and HPV16-negative head and neck cancers may harbor p53 mutations, these mutations are more common in HPV non-associated cancer (52% versus 25%) and the disruptive mutations associated with worse survival are not encountered in HPV-associated cancers (Westra et al., 2008). Overexpression of p16INK4A, induced as a consequence of the HPV-encoded E7 protein, downregulating the tumor suppressor Rb, is now known to be a clinically relevant biomarker for oncogenic HPV infection, and allows for more accurate detection of transforming HPV infection (Weinberger et al., 2006). High human papillomavirus viral load is inversely associated with p53 and p16 content (Weinberger et al., 2006).

Patients with HPV-associated cancers respond well to induction chemotherapy, combined-modality therapy, or altered-radiation fractionation, and have a lower rate of second primary cancers 2010 (Ang et al.; Fakhry et al., 2008; Rischin et al., 2009; Rischin et al.). In contrast, patients with alcohol and tobacco-induced, HPV-non-associated cancers have low cure rates even with induction chemotherapy or other forms of treatment intensification (Ang et al., 2011a; Posner et al., 2011; Rischin et al., 2010). Improved therapy for this second group of patients may depend on the development of novel agents that target the dysregulated

signaling that controls tumor cell growth, DNA repair or survival. Molecular determinants related to signaling within the epidermal growth factor receptor (EGFR) pathway have been extensively studied in SCCHN, and therapeutics specifically targeted to EGFR, such as cetuximab, are some of the most valuable agents available for treatment of SCCHN. Importantly, a recent study has specifically implicated EGFR signaling in HPV-negative SCCHN with poor prognosis (Young et al., 2011).

In this article, we provide an updated summary of therapies targeting EGFR and related proteins, emphasizing application in SCCHN. We then extensively discuss factors associated with resistance to EGFR-targeting agents, and describe new therapeutic combination approaches that are under investigation with the goal of improving management of SCCHN. Literature data published until August 1, 2011 are examined.

2. Standard of care for head and neck cancer in 2011: the central role of EGFR- and ErbB-targeted inhibitors

EGFR is a transmembrane tyrosine kinase receptor with extracellular, transmembrane, and intracellular domains. EGFR is activated by ligand binding followed either by homodimerization, or heterodimerization with another member of this type 1 receptor tyrosine kinase (RTK) family, such as ErbB2 (Her-2/neu), ErbB3, and ErbB4 (Prigent and Lemoine, 1992). Ligands for EGFR include EGF, transforming growth factor- β (TGF- β), amphiregulin, epiregulin, betacellulin and heparin-binding EGF-like growth factor (HB-EGF) (Messa et al., 1998). The EGFR extracellular ligand-binding region consists of four protein domains. Domains I and III are similar leucine-rich domains and provide the binding sites for growth factor ligands. Cooperation between domains I and III is required for high affinity binding of EGF (Lax et al., 1991). Domains II and IV are similar cysteine-rich domains. When activated, ErbB proteins are potent inducers of multiple signaling pathways that promote tumor growth and they have been a focus of intense interest for therapeutic development.

2.1. Rationale for targeting EGFR in head and neck cancer

SCCHN has proven to be sensitive to inhibition of receptor tyrosine kinases (RTK), specifically EGFR. Significantly, elevated EGFR expression detected by immunohistochemistry (IHC) is present in a majority of SCCHN, and is associated with inferior survival, radioresistance, and locoregional failure (Rubin Grandis et al., 1998) (Ang et al., 2002; Dassonville et al., 1993; Sheridan et al., 1997). Early preclinical studies revealed the anti-tumor effects of EGFR-directed monoclonal antibodies in epithelial cancer cell lines (Cohen et al., 1980; Masui et al., 1984) and confirmed that EGFR inhibition sensitizes head and neck squamous cancer cells to ionizing radiation (Balaban et al., 1996; Milas et al., 2000) (Harari and Huang, 2001; Huang et al., 1999; Kiyota et al., 2002). Inhibiting EGFR also delays the repair of chemotherapy-induced DNA damage via modulation of the DNA repair genes XRCC1 and ERCC1 (Friedmann et al., 2004; Yacoub et al., 2003). Recent studies suggest that EGFR translocates to the nucleus where it activates or represses the production of various effector proteins, such as DNA-dependent protein kinase (DNA-PK), an enzyme involved in repair of double-strand breaks of DNA caused by radiation and chemotherapy (Chen and Nirodi, 2007; Wanner et al., 2008). As outlined in detail below (sections 3 and 4), the central role of EGFR among a network of RTKs, and as master regulator of much cancer-promoting signaling, make this protein an urgent target for therapeutic development. A summary of EGFR-targeting agents currently in clinical use or development towards the clinic is shown in Table 1.

2.2. EGFR and other ErbB family-targeted inhibitors: current practice emphasizes the use of cetuximab

Thus far, cetuximab, a monoclonal antibody which targets EGFR, has been most successful in improving clinical outcomes in SCCHN. Cetuximab is a chimeric monoclonal antibody (65% human and 35% murine), constructed on an immunoglobulin (Ig) G1 framework, which targets an extracellular epitope in the EGFR ligand-binding domain (Goldstein et al., 1995). Mechanisms that contribute to the anti-tumor activity of cetuximab include interference by cetuximab with the binding of natural ligands to the receptor itself, thereby disrupting EGFR signaling pathways (Li et al., 2005). Also, cetuximab facilitates induction of receptor endocytosis and thus depletion of the targeted receptors from the cell surface (Sigismund et al., 2005). Finally, the construction of cetuximab on an IgG1 framework potentially facilitates antibody-dependent cell-mediated cytotoxicity (ADCC) via recruitment of natural killer cells and macrophages (Kurai et al., 2007; Lopez-Albaitero and Ferris, 2007). ADCC is influenced by Fc γ receptor polymorphisms. A polymorphism in Fc γ R IIIa receptor, Fc γ R IIIa-158, which is expressed on NK cells and associated with enhanced ADCC, is linked with increased cetuximab activity in SCCHN cells *in vitro* (Lopez-Albaitero et al., 2009).

In the clinical arena, data support the use of cetuximab in the setting of definitive treatment with radiation, in the first-line setting for recurrent/metastatic disease and for platinum refractory disease. The role of cetuximab when incorporated into induction chemotherapy regimens, especially in HPV-associated SCCHN is currently being studied in an ongoing Eastern Cooperative Oncology Group (ECOG) trial, E1308. Key clinical data to date include a pivotal phase III international trial, conducted by Bonner et al, in which 424 patients with locally advanced disease were randomized between definitive radiation and concurrent radiation with cetuximab (given at 400 mg per m² of body surface area loading dose followed by 250 mg per m² weekly for eight planned doses) (Bonner et al., 2006). Cetuximab plus radiation improved the median duration of loco-regional control from 14.9 to 24.4 months (p=0.005) and median survival from 29.3 to 49 months (p=0.03).

It has been of interest whether cetuximab in combination with cisplatin can improve outcomes for locally advanced SCCHN. RTOG 0522 was a large, randomized phase III trial that randomized patients to receive either concurrent accelerated radiation and cisplatin or concurrent accelerated radiation, cisplatin and cetuximab. Data presented at the 2011 American Society of Clinical Oncology (ASCO) meeting revealed that there was no difference in survival between the two treatment groups, with the hazard ratios for progression-free survival (PFS) and overall survival (OS) being 1.05 and 0.87 (p=17), respectively (Ang et al., 2011b). While 940 patients were enrolled, the study had only 84% power to detect a hazard ratio (HR) of 0.75 for the addition of cetuximab with full reporting. Thus, it is likely that the study will be underpowered even when the data are mature, in light of the good prognosis of HPV-positive patients, and the proportion of HPV-associated cancers included in the trial. Tissue for HPV analysis was not available on all patients, but among the oropharynx patients who were tested, 75% were p16 positive.

Burtneess and colleagues completed the first clinical trial (E5397) investigating the role of cetuximab in the first-line treatment of incurable advanced SCCHN (Burtneess et al., 2005). A total of 117 patients who had not received prior chemotherapy for recurrent and/or metastatic disease were randomized to either cisplatin (100 mg/m² every 4 weeks) with placebo or to cisplatin with cetuximab (400 mg/m² loading dose followed by 250 mg/m² weekly). There was a statistically significant improvement in response rate from 10% to 26% with the addition of cetuximab (p= 0.03) with a trend towards an improvement in overall survival from 8 to 9.2 months. However, the difference in survival was not statistically significant, likely due to lack of power, as well as a study design that allowed

crossover to cetuximab if patients had progressed on the placebo arm. In a much larger phase III study known as the EXTREME trial, 442 patients with advanced SCCHN who had not received prior treatment for recurrent/metastatic disease were randomized to either a platinum-containing doublet or a similar doublet with cetuximab (Vermorken et al., 2008). The chemotherapy regimen used was platinum (cisplatin at 100 mg/m² or carboplatin AUC 5 on day 1) in combination with 5-fluorouracil (1000 mg/m² on days 1–4 for a maximum of 6 cycles). Patients randomized to receive cetuximab with chemotherapy could continue to receive maintenance cetuximab until progression. Cross-over to cetuximab for those patients initially randomized to chemotherapy alone was not allowed. The addition of cetuximab showed a statistically significant improvement in survival from 7.4 to 10.1 months (p=0.036). These data established the role of cetuximab in first-line therapy for advanced SCCHN.

Three trials have established the activity of cetuximab among patients with platinum-refractory disease. In a phase II trial, 96 patients with platinum-refractory disease were treated by adding cetuximab to the platinum dose and schedule that the patients had previously failed (Baselga et al., 2005). The response rate was 10%, with a disease control rate of 53%, median time to progression of 2.79 months and overall survival of 6.01 months. In a similar phase II study, 130 patients with stable disease (SD) or progressive disease (PD) on previous platinum therapy, received treatment with cetuximab and cisplatin (Herbst et al., 2005). There were two PD cohorts: PD1 (n= 25), which had patients whose disease progressed on two cycles of protocol-specified platinum-based therapy and PD2 (n= 54), which had patients whose disease progressed within three months of any platinum-based therapy. The response rates were 18% for the SD cohort, 20% for the PD1 cohort and 6% for the PD2 cohort with median survivals of 11.7 months, 6.1 months and 4.3 months respectively. A third phase II study enrolled 103 patients actively failing platinum-based therapies and treated them with cetuximab as a monotherapy (Vermorken et al., 2007a). They reported a response rate of 12.6%, disease control rate of 46% and median overall survival of 5.84 months.

Overall, the single-agent activity of cetuximab among patients with platinum refractory SCCHN is modest with response rates consistently being 10% across multiple clinical trials. In a retrospective review of 53 patients with recurrent/metastatic disease, neither p16 expression nor EGFR amplification were associated with response. A variant of EGFR, EGFRvIII, which has a deletion of exons 2 through 7, has been described. EGFRvIII is weakly constitutively active in a ligand-independent manner. Cells that harbor this mutation are likely to be less responsive to treatment with important EGFR-targeting agents such as cetuximab (Sok et al., 2006). Interestingly, the presence of EGFRvIII seemed to be a prognostic marker that is associated with improved outcomes, irrespective of therapy. This obviously needs to be studied further in a prospective fashion (Chau et al., 2011). Resistance may arise from activation of crucial signal transduction molecules downstream from EGFR, upregulation of other receptor tyrosine kinases that signal through common mediators, altered receptor trafficking, or suboptimal immune modulation, as detailed in sections 3 and 4 of this article. Further, the ability of current dosing schedules to optimally inhibit EGFR ligand binding and downstream signaling without regard to tumor burden or receptor density is not fully studied; improved knowledge in these areas may also increase clinical response.

2.3. Emerging ErbB-family targeting agents

Overcoming mechanisms of intrinsic and acquired resistance to current generation ErbB-targeted therapies is a critical area of investigation. Next-generation agents that are being developed include antibodies, antibody-derived agents, and small molecule inhibitors.

2.3.1. Antibodies in the clinic—Like cetuximab, nimotuzumab is constructed on an IgG1 framework that potentially allows these agents to mediate ADCC via natural killer (NK) cells and macrophages. Nimotuzumab binds to EGFR on domain III, similar to cetuximab, but with lesser affinity. The clinical implications of this are unclear, given preclinical data that higher affinity antibodies can be associated with decreased tumor penetration (Adams et al., 2001). Preliminary clinical data with nimotuzumab indicate that it can be combined safely with radiation and cisplatin plus radiation (Babu et al., 2010). However, it is unknown which patient population may derive benefit from this antibody in contrast to other available monoclonal antibodies against EGFR. In one clinical trial involving nimotuzumab either with or without chemoradiation, biomarkers including expression of EGFR, pAKT, pStat3, ErbB3, and MAPK (see sections 3 and 4) were evaluated to determine if they were associated with response. Among the patients who received nimotuzumab with chemoradiation, the median survival was over 30 months versus 22 months in the control group of patients ($p=0.003$). Two EGFR antibodies were used to evaluate EGFR expression, mR3, which detects an epitope similar to nimotuzumab and a commercially available antibody, which recognized a cytoplasmic domain of EGFR. With mR3, there was a correlation between EGFR expression independent of localization and ErbB3 and MAPK expression, as well as survival among patients who received nimotuzumab and chemoradiation (Basavaraj et al., 2010) (Basavaraj et al.).

For mAb-based therapies, the development of phage-display techniques and the creation of transgenic mice that encode the human IgG locus have resulted in the ability to isolate and test fully human mAbs as one strategy to address these issues (Lonberg, 2008). Fully human mAbs are predicted to have lower levels of immunogenicity and by extension better PK and PD profiles than their chimeric and humanized counterparts, leading to more effective tumor control. This class of agents is exemplified by the anti-EGFR antibodies panitumumab (Van Cutsem et al., 2007), zalutumumab (Machiels et al., 2011), and necitumumab (Kuenen et al., 2010) that are in various stages of clinical development for EGFR-driven cancers.

Panitumumab, a fully human anti-EGFR antibody constructed on an IgG2 framework, does not mediate ADCC (Wirth LJ, 2007). In contrast to cetuximab, it is associated with a very low rate of infusion-related hypersensitivity reactions. Although approved for the treatment of colorectal cancer, panitumumab is currently being evaluated in the setting of SCCHN either as a second-line monotherapy (PRISM) or in combination with chemotherapy (PARTNER). Current data with this antibody include a phase I study of panitumumab, carboplatin, paclitaxel and radiation for locally advanced disease, which indicates that this combination is feasible (Wirth et al., 2010). In addition, preclinical data with head and neck xenografts suggest that the combination of panitumumab and radiation augments radiation-induced apoptosis as well as DNA damage, and inhibits radiation-induced activation of EGFR and downstream signaling through MAPK and STAT3 (Kruser et al., 2008).

Zalutumumab, a human IgG1 antibody targeting EGFR, has also been studied in clinical trials for patients with SCCHN. A total of 286 pretreated, platinum-refractory patients with incurable disease (recurrent or metastatic platinum-resistant SCCHN) were enrolled in a phase III trial and randomized to either zalutumumab versus best supportive care with an option of including methotrexate, which was exercised in about 75% of patients. There was a significant improvement in progression-free survival ($p=0.0012$) favoring the patients who were treated with zalutumumab and a trend to a benefit in overall survival. The decreased impact on overall survival could be a result of differences with subsequent therapy between the two groups, with 28% of patients in the control group receiving further therapy as opposed to 14% in the zalutumumab group. The study may have been underpowered because use of methotrexate in the best supportive care arm was expected to be much lower than it proved to be (Machiels et al., 2011). Finally, necitumumab is being investigated in a

number of EGFR-driven cancers, including in a phase III trial of squamous non-small-cell lung cancer (NSCLC) in combination with chemotherapy (SQUIRE).

2.3.2. Next-generation antibody derivatives—The modular nature of the IgG structure, combined with improved antibody engineering techniques and manufacturing capabilities, has facilitated the development of a large variety of bispecific antibodies (bsAbs), examples of which are depicted in Figure 1. The development and testing of bsAbs is being driven by two distinct approaches for improving upon current mAb-based therapies. The first approach is based on the hypothesis that simultaneous targeting of two disease mediators, such as the EGFR and IGF1R (see section 3), with a bsAb will more effectively block critical signaling pathways leading to enhanced tumor control. This hypothesis was borne out in preclinical testing of two bsAbs, an IgG-like Di-diabody that was generated from the variable domains of the anti-EGFR IMC-11F8 (necitumumab) and anti-IGF-1R IMC-A12 (cixutumumab) (Lu et al., 2005) and an IgG-scFv created from a human anti-EGFR (M60-A02) FAb and a stability-enhanced variant of the anti-IGF-1R scFv BIIB5 (Dong et al., 2011). Both of the anti-EGFR/anti-IGF1R bsAbs were capable of simultaneously inhibiting IGF and EGF-stimulated signaling *in vitro* and slowing tumor growth in xenograft models that express both receptors.

Likewise, the heterodimerization of ErbB family members and the role of ErbB3 in mediating resistance to ErbB-targeted inhibitors (Baselga and Swain, 2009) underlie the development of two agents currently in clinical trial, the anti-EGFR/anti-ErbB3 IgG MEHD7945A (Schaefer, 2010) and the anti-ErbB2/anti-ErbB3 bispecific single-chain Fv (bs-scFv) MM-111 (Denlinger et al., 2010). In contrast to other bsAbs that use distinct variable domains to bind to each target antigen, the variable domains comprising MEHD7945A were engineered to bind with high affinity to both EGFR and ErbB3 on non-homologous epitopes (Bostrom et al., 2009). This dual-specificity IgG is capable of blocking ligand-dependent activation of both EGFR and ErbB3 and has preclinical activity against multiple EGFR-driven cancers, including SCCHN. MEHD7945A is currently in phase I clinical trials in the setting of SCCHN, pancreatic, colorectal and non-small-cell lung cancers. The bs-scFV MM-111 uses human serum albumin as a linker between the anti-ErbB2 and anti-ErbB3 scFv to improve the PK of the molecule. Analogous to the immune modulatory antibodies described below, MM-111 does not treat cancers by inhibiting ErbB2 signaling; instead, it takes advantage of the high level of ErbB2 overexpression that is often seen in breast and gastric cancers to target the antibody to the tumor cells and deliver the therapeutic anti-ErbB3 arm of the antibody to the tumor cell. This agent is currently in a series of phase I and phase II clinical trials as both a monotherapy and in combination with standard-of-care agents. The modular nature of MM-111 could easily be adapted to the setting of SCCHN and other EGFR-driven cancers by substituting an EGFR-targeting arm in place of the ErbB2 arm of MM-111.

The second approach driving the development of bsAbs is based on the hypothesis that bsAbs can be engineered to redirect immune effector cells to kill tumor cells by promoting ADCC, thus bypassing the common resistance mechanisms associated with signal transduction inhibitors. Although useful for any class of effector cells, this approach is particularly intriguing in the context of redirecting cytotoxic T cells, which are the most potent killer cells of the immune system. This class of immune effector cells is highly abundant, can both proliferate and kill multiple times upon activation and are known to infiltrate tumors. However, they fail to express Fcγ receptors so cannot directly participate in antibody-dependent cellular cytotoxicity mechanisms elicited by classic IgG therapies (Chames and Baty, 2009).

In this approach the bsAb is composed of a tumor-targeting arm that is specific for a tumor-associated antigen and an immune effector arm that binds to an activation receptor, such as CD3, on the surface of T cells. This approach is exemplified by the Bispecific T cell Engager (BiTE, Micromet) and Triomab (Trion pharmaceuticals) platforms that are currently in various phases of clinical development. Both platforms rely on anti-CD3 arms to recruit T cells. Blinatumomab (MT103) is an anti-CD19/anti-CD3 bs-scFv that is being tested in the setting of B cell lymphomas (Bargou et al., 2008) and MT110 is an anti-EpCAM/anti-CD3 agent being tested in phase I trials in the setting of solid tumors (Fiedler et al., 2010). The Triomab platform takes advantage of selective heterodimerization of modified Fc domains to create bispecific IgGs. The anti-EpCAM/anti-CD3 antibody catumaxomab is currently approved by the EU regulatory agency for treatment of malignant ascites. The anti-ErbB2/anti-CD3 antibody ertumaxomab (Jager et al., 2009) is in phase II trials in both the EU and US. Both the BiTE and Triomab platforms are easily adaptable to other malignancies, such as SCCHN, by incorporation of the appropriate targeting arms. Preclinical testing of an anti-EGFR/anti-CD3 bispecific antibody has been described (Reusch et al., 2006).

2.3.3. Small molecule inhibitors—Small molecule tyrosine kinase inhibitors (TKIs) are typically quinazoline-derived synthetic molecules that block the adenosine triphosphate (ATP) binding site of the intracellular tyrosine kinase domain of EGFR and other tyrosine kinase receptors. While some are specific for EGFR (gefitinib, erlotinib), others target other receptors as well, such as ErbB2 (e.g., lapatinib), and HER1/ErbB2/HDAC (CUDC-101). In the past, small-molecule EGFR-targeting inhibitors have not been found to be highly active in SCCHN, in spite of their clear ability to induce striking clinical benefits in other EGFR-associated tumors. However, several clinical trials are currently investigating the use of small-molecule EGFR-targeted inhibitors in specific patient populations, or in combination therapies.

In a phase II study, the oral EGFR TKI gefitinib yielded a response rate of 10.6% in a population of patients with recurrent/metastatic disease, which is comparable to the single agent activity of cetuximab, but still modest (Cohen et al., 2003; Vermorken et al., 2007b). Also, a study by the Eastern Oncology Cooperative Group (ECOG 1302), in which patients were randomized to docetaxel versus docetaxel plus gefitinib, reported a statistically significant increase in time to progression in the latter arm (Argiris et al., 2009). Erlotinib has been evaluated in SCCHN as well, with an objective response rate (ORR) of 4.3% and OS of 6 months (Soulieres et al., 2004). An ongoing trial at Fox Chase Cancer Center is investigating the addition of erlotinib to a chemotherapy and cetuximab backbone in metastatic/recurrent disease. In preclinical studies, lapatinib exhibited anti-tumor activity in head and neck cell lines as a single agent and in combination with cisplatin and paclitaxel (Kondo et al., 2010). However, in a phase II trial for recurrent/metastatic disease, there was little single-agent activity with lapatinib with no objective responses and a PFS of 1.7 months (Abidoye et al., 2006). Phase I data combining lapatinib with cisplatin at 100 mg/m² and radiotherapy to 66–70 Gy, indicated that a dose of lapatinib of 1500 mg was tolerable and yielded an ORR of 81%. Toxicities were as expected and included mucositis, dermatitis, lymphopenia and neutropenia (Harrington et al., 2009).

In a follow-up randomized phase II trial, 67 patients were treated with either chemoradiation versus lapatinib and chemoradiation followed by maintenance lapatinib (Harrington et al., 2010). Only 28% of tumors were p16 positive, suggesting that this was a predominantly HPV-negative population. There was an improvement in progression-free survival from 12 to 20 months. Thus, as lapatinib is studied further in combination with chemoradiation, consideration of activity among p16 negative tumors is warranted. Irreversible inhibitors of EGFR are also being developed and studied in NSCLC and SCCHN. For instance, afatinib

(BIBW2992), an anilino-quinazoline derivative, is a dual inhibitor of EGFR and ErbB2 (Minkovsky and Berezov, 2008). This agent is being studied in two ongoing trials for SCCHN. In one, the goal is to evaluate its role as adjuvant therapy after definitive chemoradiation (NCT01345669). In another ongoing trial for recurrent/metastatic disease, patients will either be randomized to afatinib or methotrexate (NCT01345682).

CUDC-101 is a novel potent inhibitor of EGFR, HDAC and ErbB2 and has been shown to have anti-tumor activity in head and neck cancer xenograft models (Cai et al., 2010). CUDC-101 is also being actively investigated in combination with chemoradiation for patients with HPV-negative tumors. The rationale of this approach is that these more treatment-resistant tumors would benefit from targeting several pathways simultaneously. Thus, overall, there are many emerging novel agents, both antibodies and small molecules, which are the subject of ongoing studies for SCCHN.

2.4. Mutations in EGFR affecting therapeutic resistance

A number of mutations have been identified in the EGFR tyrosine kinase domain (TK) in NSCLC tumors (Pao and Chmielecki, 2011). Kancha et al. evaluated the growth factor dependence of 30 previously observed EGFR TK mutations in NSCLC and found that 25 of them were independent of growth factor (Kancha et al., 2009). Of these 25, all but one were sensitive to gefitinib and erlotinib, but with highly varying IC₅₀s (7 nM to 505 nM), while the wild-type IC₅₀ has been reported to be 5–50 nM (Pedersen et al., 2005). Thus, the reported increase in sensitivity of some tumors with mutated EGFR to gefitinib may not be due to higher activity of the inhibitor against the altered EGFR enzyme, but rather higher dependence of the mutant tumors on EGFR kinase activity. Of the TK mutations assessed, only the T790M variant of EGFR resulted in kinase activity resistant to both drugs (IC₅₀>2000 nM). This mutant has been observed in a number of NSCLC studies as a secondary mutation in EGFR associated with acquired resistance to gefitinib (Kobayashi et al., 2005; Pao et al., 2005). In a X-ray crystallographic structure of EGFR TK with gefitinib, the wild-type threonine was in direct contact with the bound inhibitor (Yun et al., 2007). However, Murray et al. found no T790M mutations in 19 gefitinib-treated SCCHN cases (Murray et al., 2010). Mutations that affect the binding site of cetuximab or other monoclonal antibody treatments do not seem to have been observed to date.

In SCCHN itself, mutations in EGFR are relatively rare (Hama et al., 2009; Machiels and Schmitz, 2011). Lee et al. found EGFR mutations in only 3 of 41 larynx, tongue, and tonsil tumor samples in Korean patients (Lee et al., 2005). All three contained an in-frame deletion of 5 amino acids (positions 746–750, sequence ELREA). This sequence comprises the last two residues of the last beta sheet strand of the N-terminal domain of the EGFR kinase domain and the first three residues of the 5 residue loop that connects to the C-helix. SRC kinase has a three residue deletion in this region (relative to EGFR) with one less turn in the helix and a shorter distance between the beta sheet and the C-helix, providing a good template for comparison with EGFR. It is likely that EGFR kinase tolerates the deletion observed in these patients by shortening the helix by at least one full turn and a subsequent shift in some residues into the beta sheet strand and an adjustment of the C-helix position, resulting in a constitutively active kinase. The superposition of EGFR TK and SRC TK is shown in Figure 2A.

Hama et al. found five different EGFR mutations in 6 of 82 SCCHN patients (7%) (Hama et al., 2009). One of these, L858R, has been found in lung cancer patients and is considered an activating mutation of EGFR kinase function (Won et al., 2011). It immediately follows the DFG sequence at the N-terminus of the activation loop. Another, V765G, changes a hydrophobic residue on the C-helix that interacts with the C-terminal domain; removal of this group would alter the interaction of the N and C terminal domains, which regulates

kinase activity. Loeffler-Ragg et al. found only one missense mutation in 100 head and neck tumor samples (Loeffler-Ragg et al., 2006). This mutation, K745R, involves a lysine residue that binds the alpha phosphate of ATP. A change at this position is highly likely to alter kinase function, potentially as an activating mutation. Schwentner et al. identified the same mutation in 3 of 126 SCCHN patients, as well as the G796S in 2 patients (Schwentner et al., 2008). This residue is in contact with ATP. These mutated residues (L858, V765, K745, G796) are shown in Figure 2B. All four of them are in proximity of ATP and/or the interface between the N and C terminal lobes of the kinase.

EGFR variant III (EGFRvIII) involves a deletion of domain I and more than half of domain II (encoded by exons 2–7), as shown in Figure 3. Domain I participates in ligand binding and domain II participates in homo and heterodimerization. EGFRvIII is weakly constitutively active in a ligand-independent manner. EGFRvIII has been found in up to 40% of SCCHN tumor samples (Chau et al., 2011). Tinhofer et al. found that 17% of 47 metastatic SCCHN after cetuximab treatment had EGFRvIII mutations and this was associated with a decreased disease-free state (Tinhofer et al., 2011). Cetuximab binds to domain III of EGFR, and is therefore also able to bind to EGFRvIII, which retains the entirety of domain III (Jutten et al., 2009). Interestingly, they found that instead of inhibiting EGFR activity, cetuximab activates EGFRvIII phosphorylation in glioma cells. Given the relevance of EGFRvIII expression in SCCHN response to treatment, more study is merited (Chau et al., 2011).

3. Targeting ErbB-collaborating RTKS and other transmembrane receptors in head and neck cancer

The oncogenic role of the ErbB proteins reflects their ability to activate a series of effector cascades (summarized in section 4) that collectively promote tumor growth. A complicating factor for treatment of head and neck cancer based on inhibition of ErbB proteins is that additional RTKS or transmembrane receptor proteins are coupled to some of the same effectors that interact with ErbB proteins. Of these, IGF1R and c-MET are two of the best-documented sources of treatment resistance in HNC. Interactions between EGFR and these other transmembrane receptors is shown in Figures 4A–B.

3.1. IGF1R

It has long been known that EGFR signaling depends in part on functional co-signaling by the insulin growth factor 1 receptor (IGF1R) (Coppola et al., 1994) (Figure 4A). The receptor forms a tetramer after activation by its ligands IGF-1 and IGF-2. These ligands are sequestered by IGF binding proteins, which thus function as IGF1R antagonists. More than 90% of IGF-1 is in a complex with IGFBP-3 under normal conditions. IGF1R downstream effects include transactivation of EGFR, activation of the Ras/Raf and phosphatidylinositol-3-kinase (PI3K) signaling pathways, enhanced survivin expression, cell proliferation, altered cell adhesion, motility properties and impaired apoptosis (Pollak, 2008; van der Veeke et al., 2009). Finally, IGF-1 induces vascular endothelial growth factor (VEGF) secretion from head and neck cell lines, such as SCC-9 cells (Oh et al., 2008; Slomiany et al., 2006).

In 2002, upregulation of IGF1R was shown to compensate for inhibition of EGFR in glioblastoma cells, based on the ability of IGF1R to separately support the activity of PI3K (Chakravarti et al., 2002). Subsequently, IGF1R activation of its substrate IRS1 was observed in gefitinib-resistant A431 cell lines, reflecting downregulation of the IGF1R-inhibitory proteins IGFBP-3 (Guix et al., 2008). IRS1 was found to be a hub for a feedback process in which inhibition of EGFR or IGF1R individually resulted in activation of the other. As

predicted by this result, dual inhibition of ErbB family proteins and IGF1R resulted in synergistic inhibition of tumor cell growth in various models (Buck et al., 2008; Huang et al., 2009). These results have also suggested the merit of exploring dual inhibition of these pathways in the clinic.

3.1.1. IGF1R in head and neck cancer: tumor-associated expression changes, and clinical targeting—Activation of the IGF1R signaling pathway is strongly associated with solid tumors of the head and neck. Expression of IGF1R is detected in squamous cell carcinoma cell lines and Western blotting detects elevated IGF1R protein expression in the majority of head and neck tumors (Barnes et al., 2007). The clinical relevance of this finding is highlighted by the role of the IGF-1 pathway in development of second primary tumors (SPT) in head and neck cancer survivors. Investigators of the Retinoid Head and Neck Second Primary Trial analyzed IGF-1 and IGFBP-3 serum levels in pre-treatment specimens from 80 participants who developed SPT, and 160 participants without SPT. Serum levels of IGF-1 were significantly correlated with IGFBP-3 levels. Patients with higher IGF-1 levels and higher IGF-1/IGFBP-3 ratios had significantly higher risk of SPT; after adjustment for smoking status and treatment assignment, the OR for SPT in patients with IGF-1 levels above 104.25 ng/ml was 3.66. IGFBP-3 displayed a biphasic relationship with risk, with the lowest risk of SPT observed in patients with midrange IGFBP-3 levels and higher rates of SPT in those with low or high levels (Wu et al., 2004).

Introduction of siRNA specific to IGF1R inhibits growth of IGF1R-expressing head and neck cancer cell lines, without inducing apoptosis. IGF-induced ERK phosphorylation can be inhibited with A12 (cixutumumab), an IGF1R directed monoclonal antibody. This antibody also causes G₁ cell cycle arrest both in IGF1R high- and low-expressing head and neck squamous cell carcinoma cell lines (Barnes et al., 2007). Signaling from activated IGF1R has been seen as a potential mechanism of resistance to EGFR inhibition in other solid tumors, and thus it is of interest that either IGF or EGF can induce EGFR/IGF1R heterodimerization in TU159 head and neck squamous cell carcinoma cells (Barnes et al., 2007). TU159 xenografts regress after exposure either to cetuximab or to A12, with an additive effect when cetuximab and A12 are given together. A12 enhances radiosensitivity of head and neck squamous cell carcinoma cell lines and xenografts in an additive or sub-additive fashion (Allen et al., 2007; Riesterer et al., 2011).

Inhibitors of IGF1R that have entered the clinic include both monoclonal antibodies and tyrosine kinase inhibitors; however, neither the safety nor the efficacy of these agents for head and neck cancer patients is clear at this time. Figitumumab (CP751,871) was tested in a phase II trial for recurrent/metastatic head and neck cancer terminated early for lack of efficacy [<http://clinicaltrials.gov/ct2/show/NCT00872404>; accessed June 28, 2011.] A phase III trial of this agent in unselected non-small cell lung cancer was discontinued because of lack of efficacy; subsequent analysis of serum samples from that trial established that the addition of figitumumab to chemotherapy was beneficial only in patients with elevated free pre-treatment IGF-1 (Gualberto et al., 2011). Trials of A12 combined with cetuximab are not yet recruiting. Despite the clear evidence that IGF1R represents a promising target in head and neck cancer, the ultimate utility of targeting IGF1R signaling remains uncertain. Defining the biosignature of potentially responsive patients before embarking on trials of IGF1R-directed inhibition in head and neck cancer may be necessary, to avoid a repetition of the experience in non-small cell lung cancer.

3.2. c-MET

c-MET is a transmembrane tyrosine kinase receptor for the hepatocyte growth factor (HGF, scatter factor), encoded by the MET gene on chromosome 7q31. Important downstream

signals of c-Met overlap with transducers of EGFR signaling, and include p44/p42 mitogen-activated protein kinase (MAPK), PI3K/AKT, STAT3 and PLC γ (Figure 4B). c-Met signaling also results in release of potent cytokines such as IL-8. HGF/c-Met signaling is also associated with a number of hallmarks of malignancy, notably increased cell motility, invasion and angiogenesis. Expression of c-Met has been associated with invasiveness across a number of tumor types, and c-Met signaling has been implicated in resistance to EGFR inhibition in non-small cell lung cancer (Jemal et al., 2007). c-Met is currently being investigated not only as a potential biomarker, but also as a potential therapeutic target in SCCHN.

In some tumors that have acquired resistance to EGFR-targeted inhibitors, MET maintains the activation of EGFR effector pathways based on amplification of the MET protein (Engelman et al., 2007; Sequist et al., 2011; Turke et al., 2010). Phosphoproteomic analysis has shown that MET activation triggers activity in the ErbB2 and ErbB3 RTKs, and also revealed a large set of common targets that support tumor growth that are comparably activated by EGFR or MET (Guo et al., 2008). Experimentally, overexpression of the MET ligand, HGF, has been shown to similarly override the effect of EGFR inhibition by cetuximab in colorectal cancer (Liska et al., 2011). A study of NSCLC patients has found increased expression and activation of MET associated with primary resistance to EGFR inhibitors (Benedettini et al., 2010) and cell line studies have shown similar effects in opposing the action of EGFR/ErbB2 inhibitors (Agarwal et al., 2009). Cumulatively, these and other data strongly support the idea that dual inhibition of MET and ErbB family members might offer a productive strategy for enhancing the activity of ErbB-targeted inhibitors. Strategies for inhibiting MET under exploration include the use of antibody inhibitors of MET or its ligand, HGF, or small molecule inhibitors of MET kinase (reviewed recently in (Comoglio et al., 2008)).

3.2.1. Involvement of c-MET in head and neck cancer—In head and neck cancer, c-Met overexpression was first reported by Seiwert et al in 84% of a series of 121 specimens (Seiwert et al., 2009). These findings were confirmed in a subsequent series of oral squamous tumors: low level c-Met expression was confined to basal epithelium in normal oral mucosa, but the majority of 53 cancers expressed c-Met in cytoplasm; 72% also displayed nuclear c-Met, predominantly at the invasive front. In this small series, no relationship between c-Met expression and prognosis was discerned (Brusevold et al., 2010). Similarly, among 49 patients with recurrent/metastatic head and neck cancer treated at the Princess Margaret Hospital, 31 displayed 3+ staining for c-Met. There was no relationship with outcome in this series; however, response rates and median survival times were low in these patients (Chau et al., 2011). Two somewhat larger series from Asia correlated c-Met expression with higher lymph node stage and significantly shortened survival (Kim et al., 2010; Zhao et al., 2011). Utilizing human papillomavirus-negative squamous carcinoma lines, Knowles et al. demonstrated c-Met but not HGF expression (Knowles et al., 2009). Addition of HGF induced c-Met phosphorylation, leading to activation of AKT and MAPK, release of IL-8, and increased tumor cell migration and proliferation. These responses were blocked with the MET inhibitor SU11274.

c-Met expression is regulated by EGFR and hypoxia-inducible factor alpha (HIF1- α). In a non-small cell lung cancer model, inhibition of mutated EGFR decreases MET RNA, and knock-down of EGFR resulted in reduced c-Met expression and activation (Xu et al., 2010). EGF stimulation triggered a rise in phospho-MET by 30 minutes, consistent with a direct effect of EGFR signaling in activating c-MET (Jemal et al., 2007). Independent of the contribution of upstream factors, c-MET activation can result from the point mutation Y1253D, and this has been identified in 14% of a series of 152 head and neck cancers (Ghadjar et al., 2009). Seiwert et al also identified MET mutations in 13% of tumors and cell

lines, with mutations in the semaphorin, juxtamembrane and tyrosine kinase domains (Seiwert et al., 2009). As had previously been reported for non-small cell lung cancer, resistance to EGFR inhibition is associated with increased c-MET expression. A high-throughput antibody array analysis of receptor tyrosine kinases was performed to compare cetuximab-sensitive parental lines with cetuximab-resistant lines, and demonstrated differential, increased expression of ErbB2, ErbB3 and c-MET in the resistant sub-lines. In resistant lines, immunoprecipitation indicated that EGFR displayed increased heterodimerization with ErbB2, ErbB3 and c-MET as compared to the cetuximab-sensitive cells (Wheeler et al., 2008).

Aberrantly high expression of HGF has also been reported in head and neck cancers. Immunostaining for HGF was used to classify 127 endemic nasopharynx cancers as HGF high or low expressing, with 54% demonstrating high tumoral HGF and 80% high stromal HGF expression (Xie et al., 2010). High HGF expression in this series correlated with higher expression of IL-8, as well as significantly worse survival. Those patients co-expressing HGF and IL-8 at high levels demonstrated the lowest median survivals. The finding of improved locoregional control when tirapazamine, a cytotoxic agent which is preferentially active in hypoxic cells, was added to chemoradiation in p16-negative (HPV non-associated) oropharynx cancer patients, but not in p16-positive patients, raises the question of whether hypoxia is more prevalent in HPV non-associated head and neck cancer, and whether MET expression, regulated by HIF1- α , may constitute a more important target in HPV non-associated cancers. No significant differences in tissue pO₂ or in IHC for carbonic anhydrase IX (CAIX) have been reported between HPV-negative and HPV-positive tissues, but ongoing biomarker analysis of the tirapazamine study will include determination of HGF and IL-8 levels (Kong et al., 2009; Rischin et al., 2010).

3.2.2. c-MET Inhibitors in the clinic—Foretinib (GSK1363089) is a multi-targeted kinase inhibitor of c-MET and the pro-angiogenic receptor VEGFR2. A 40-patient phase I study reported a maximum tolerated dose of 3.6 mg/kg. Dose-limiting toxicities were grade 3 elevations in aspartate aminotransferase and lipase. Hypertension, fatigue, diarrhea, vomiting, proteinuria, and hematuria were also observed. There were two objective responses and more than half of the patients treated had disease stabilization. MET phosphorylation was inhibited and proliferation markers decreased in a subset of tumors biopsied after drug exposure (Eder et al., 2010; Qian et al., 2009). A phase II study of foretinib in head and neck cancer has completed enrollment but not yet been reported.

ARQ 197 is an orally administered small molecular inhibitor of c-MET. In phase I trials, it was well tolerated, with dose-limiting toxicities of grade 3 fatigue, mucositis, palmar-plantar erythrodysesthesia, and hypokalemia; febrile neutropenia was also observed in this monotherapy study. The recommended phase II dose is 360 mg twice per day. Pharmacodynamic studies demonstrated post-treatment decreases in phosphorylated c-MET, total c-MET, and phosphorylated focal adhesion kinase (FAK), and increased terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL) staining in tumor biopsies. Fourteen of 51 patients achieved stable disease (Yap et al., 2011).

AMG102 is a fully humanized neutralizing antibody to HGF. Dose escalation in the phase I trial continued to 20 mg/kg without defining the maximum tolerated dose. The most common adverse events were fatigue, anorexia and nausea (Gordon et al., 2010). Plasma total HGF levels increased with increasing dose and duration of AMG102 treatment, perhaps indicative of decreased degradation of HGF when AMG102 bound, and 16 of 40 patients had disease stabilization. The clinical experience to date suggests that the available c-MET and HGF inhibitors are tolerable, with side effect profiles that may permit combination with

EGFR inhibitors or chemotherapy in some cases. These agents are good candidates for further testing in both HPV non-associated locally advanced SCCHN, and in cisplatin-refractory recurrent/metastatic disease.

3.3. TGF- β

The transforming growth factor β (TGF β) signals through a transmembrane receptor and a series of intermediate proteins to control the transcription of genes such as E-cadherin that control epithelial-mesenchymal transition (EMT), proliferation, differentiation, and survival (Figure 4A). The TGF β pathway has been reported to have complex activity in tumors, with activation of the pathway inhibiting early stages of proliferation dependent on ErbB genes, but also promoting invasion and metastasis at later stages of tumor growth (reviewed in (Inman, 2011)). In some tumor types, such as head and neck cancers, the TGF β cascade has been proposed to be predominantly tumor-suppressive, based on the frequent loss of the TGF β RII gene encoding the TGF β receptor, and multiple important signaling effectors (SMAD2-4) through chromosome 18q deletions and mutations (discussed in (Leemans et al., 2011)). However, the situation is complicated by the fact that the TGF β 1 ligand is upregulated in many head and neck cancers in a compensatory reaction to inhibition of the core pathway and other genetic changes (Inman, 2011), and conditions the tumor microenvironment in a way that promotes tumor growth. Further, loss of TGF β RII also has been reported to activate EGFR-STAT signaling, and otherwise activates signaling pathways relevant to head and neck tumors (discussed in (Connolly and Akhurst, 2011; White et al., 2010)), while downstream intermediates in the TGF β pathway such as RUNX3 have also been found to act oncogenically in this disease (reviewed in (Kudo et al., 2011)). A recently emerging theme has been the realization that this pathway is important for the maintenance of tumor stem cell populations (Asiedu et al., 2011; Mani et al., 2008; Naka et al., 2010). A variety of approaches to modulate TGF β pathway signaling are moving through preclinical and clinical testing, with some data indicating efficacy in eliminating tumor stem cell populations (Anido et al., 2010; Connolly et al., 2011; Korpil and Kang, 2010). While the complexity and apparent evolution of the role of TGF β signaling during tumor progression indicate that patient selection for inhibitors targeting this pathway will not be trivial, inhibition of this pathway may prove of significant clinical benefit in invasive, later stage tumors.

4. Alterations in the RTK signaling landscape as a basis for therapeutic resistance

Signals originating with stimulation of the ErbB and other RTKs propagate downstream, result in the activation of a number of discrete effector pathways. The direct effector pathways responding to EGFR stimulation are some of the best-studied response cascades in mammalian biology. For some cancer types, expression changes and mutational activation affecting proteins in these effector cascades have been demonstrated to confer resistance to targeting upstream signaling components such as EGFR, with K-Ras mutation limiting the efficacy of cetuximab in colorectal cancer a notable example (Khambata-Ford et al., 2007). Although relatively few such mutations have been identified in SCCHN, it is nevertheless possible that changes in the activity states of these effector proteins may contribute to drug resistance. Beyond canonical, direct ErbB effectors, additional signaling proteins are increasingly appreciated as providing input that modulates ErbB-dependent signaling, or can compensate for the reduced ErbB signaling that occurs under conditions of drug inhibition. Because of these supporting roles, proteins operating in such lateral pathways may provide alternative targets for drug inhibition that can enhance ErbB-directed targeted therapies, and biomarkers for response to these therapies. The regulatory processes discussed below have been comprehensively reviewed in recent years (Brand et al., 2011; Leemans et al., 2011;

Machiels and Schmitz, 2011; Pao and Chmielecki, 2011; Ratushny et al., 2009; Sorkin and Goh, 2009; Wheeler et al., 2010). Here, we provide a brief overview as context for clinical trials of novel agents in SCCHN: Figures 4A–C illustrate the signaling relations discussed.

4.1. Direct effectors

The C-terminal intracellular tail of EGFR contains a number of tyrosines that become trans-phosphorylated upon EGFR dimerization and activation. Additional tyrosine phosphorylations (on Y⁸⁴⁵ and Y¹¹⁰¹) are added by SRC family kinases as part of the activation process. Proteins binding to these sites or to other motifs on activated EGFR include transducers of pro-proliferative and anti-apoptotic growth signals: phospholipase C gamma (PLC γ), the adaptor proteins SHC, NCK, and CRK, STAT transcription factors, and the p85 subunit of phosphoinositol-3-kinase (PI3K). Each of these direct interactions initiates signaling processes that collaborate to support EGFR-dependent oncogenic transformation. Mutations or expression changes affecting proteins in these direct effector pathways have the potential to provide sources of therapeutic resistance, by overriding inhibition of EGFR or other upstream RTKs. Specific microenvironments within tumors can also directly activate these effectors, supporting resistance and aggressive tumor behavior: for instance, pockets of hypoxic cells in a subset of EGFR-overexpressing tumors activate EGFR and downstream targets such as PLC γ and AKT (Wang and Schneider, 2010).

4.1.1. PLC γ —In head and neck cancer, primary tumors express higher levels of total and phosphorylated PLC γ than do neighboring mucosal cells, and inhibition of PLC γ reduces EGFR-dependent tumor cell migration and invasion (Thomas et al., 2003). The biological consequences of PLC γ activation are two-fold (Figure 4C). First, PLC γ cleaves phosphatidylinositol 4,5-bisphosphate (also known as PtdIns(4,5)P₂, or more simply PIP₂) at the plasma membrane, resulting in the production of the second messengers diacyl glycerol (DAG) and inositol 1,4,5-triphosphate (IP₃). DAG activates members of the protein kinase C (PKC) family at the membrane (recently reviewed in (Rosse et al., 2010)), with these proteins variously enhancing cell polarization, migration and invasion by enhancing the activity of MET and integrins, and promoting cell survival. IP₃ diffuses through the cytoplasm, where one of its more important activities is the binding of an IP₃ receptor to trigger Ca²⁺ ion release from intracellular stores in the endoplasmic reticulum (ER). Increased intracellular Ca²⁺ activates calmodulin II kinase (CAMIIC) and calcineurin, and directly binds and induces conformational changes in other proteins to regulate their activity. Cumulatively, the perturbed Ca²⁺ signaling that is common in cancer cells also supports the cell cycle progression and survival of these cells (reviewed in (Roderick and Cook, 2008)).

Overexpression and/or hyperphosphorylation of specific PKC isoforms (e.g. PKC ϵ , PKC δ) are observed in a number of cancers, and considered predictive markers for poor disease outcomes (Clark et al., 2003; Toton et al., 2011). There have been some efforts to target PKC family members via strategies including small molecules, inhibitory peptides, or antisense, with this effort largely still at the pre-clinical stage (reviewed in (Bosco et al., 2011)). Given the complexity and sometimes opposing actions of different PKC isoforms, the selective expression of different family members in different cancer subtypes, and the difficulty in designing inhibitors targeting discrete isoforms, more work remains to be done before developing an effective strategy to exploit these proteins clinically.

4.1.2. PI3K/PTEN—Phosphoinositol-3-kinase (PI3K) plays an essential role in transmitting pro-survival and pro-growth signals in tumor cells (Figure 4C). There are multiple isoforms of a larger family of PI3K related proteins; of the three defined classes, Class I PI3Ks are most relevant to cancer (Zhao and Vogt, 2008). Each functional PI3K protein is a heterodimer, consisting of an 85 kD regulatory subunit and a 110 kD catalytic

subunit. In normal cells, the p85 regulatory subunit binds to multiple phosphotyrosine sites on the EGFR C-terminal domain, recruiting and activating the p110 subunit to catalyze the conversion of PIP2 to phosphatidylinositol 3,4,5-trisphosphate (PIP3). This activity is opposed by the phosphatase PTEN, which cleaves PIP3 back to PIP2. Together, the balance of PI3K and PTEN activity controls the accumulation of PIP3 at the membrane. A concentrated patch of PIP3 at the plasma membrane provides a docking site for proteins containing pleckstrin homology (PH) domains, one of the most important of which is the kinase AKT. Association with PIP3 at the plasma membrane allows AKT to become activated by phosphorylation by PDK1 (Alessi et al., 1997). Active AKT phosphorylates and inhibits TSC2 (activating protein synthesis through Rheb and mTOR), inactivates GSK3 β , FOXO1, BAD, and BIM (to stimulate cell cycle progression and block apoptosis), and promotes GLUT4 trafficking to the plasma membrane, enhancing glucose metabolism (Gonzalez and McGraw, 2009).

Common mutations in cancer include those that catalytically activate the PIK3CA p110 α catalytic subunit, and those that result in loss of activity of PTEN. Both of these events result in activation of PI3K and AKT signaling, but are not completely equivalent, because of additional activities unique to PI3K or PTEN. For example, tumor cells with mutated PTEN have constitutively activated JNK signaling, independent of PTEN regulation of AKT (Vivanco et al., 2007). These mutational changes can directly affect the response of cells to EGFR-targeted inhibitors. A subset of NSCLC that developed resistance to small molecule inhibitors acquired novel activating mutations in PIK3CA (Ludovini et al., 2011; Sequist et al., 2011). Similarly, mutation of PIK3CA confers resistance to monoclonal antibody therapeutics targeting EGFR in colorectal cancer, particularly when combined with mutational activation of KRAS (Jhawer et al., 2008; Sartore-Bianchi et al., 2009a; Sartore-Bianchi et al., 2009b). Loss of PTEN has also been associated with less response to cetuximab in some cancers, such as colorectal (Laurent-Puig et al., 2009). Based on the importance of this signaling axis, development of drugs to inhibit the cancer-relevant Class I alpha isoforms of PI3K has been of considerable interest. Pan-isoform-directed compounds such as NVP-BEZ235 (a dual PI3K/mTOR inhibitor) and GDC-0941 are moving through clinical assessment, and show promise, particularly in combination approaches (LoPiccolo et al., 2008; Faber et al., 2009; Raynaud et al., 2009). The recently described CH5132799 is selectively active against mutant and wild type PIK3CA, and showed significant activity in xenografts (Tanaka et al., 2011). The decision of whether to pursue a strategy of selective versus broad inhibition of PI3K may depend on the specific genetic composition of individual tumors. For instance, PTEN-deficient tumors have been shown to become dependent on p110 β rather than p110 α (Wee et al., 2008), and p110 β -directed inhibitors were more active in this subset of tumors (Edgar et al., 2010).

Resistance to EGFR inhibition with cetuximab has been identified in patients with colon cancers bearing KRAS mutations or loss of PTEN. Although the COSMIC database reports KRAS mutations in only 3% of head and neck cancers, HRAS mutations may be present in as much as 10% of these tumors, and PI3K mutation and PTEN loss are also (Forbes et al., 2008). Thus, further study of samples from randomized trials of cetuximab in head and neck cancer is warranted to discover whether similar predictors of cetuximab resistance can be identified.

4.1.3. SHC, GRB2, Ras, and beyond—A canonical effector pathway downstream of EGFR consists of a chain of adaptors including SHC and GRB2, recruiting the GTP-GDP exchange factor SOS to activate Ras (Figure 4B). GTP-bound Ras proteins bind and activate many effectors, including RAF (atop the MEK1/ERK/ETS signaling cascade), RAL, and PI3K (through direct interaction with the p110 α subunit). Because the importance of the EGFR-Ras connection has long been appreciated (Aroian et al., 1990; Han et al., 1990;

Lowenstein et al., 1992), investigations of Ras and its binding partners as modulators of EGFR signaling have been extensively investigated and reviewed (Brand et al., 2011; Vakiani and Solit, 2011). Interestingly, although activating mutations in Ras (KRAS and HRAS) and BRAF have been found to be a predominant source of resistance to EGFR-targeting agents in multiple tumor types, these mutations are relatively rare in head and neck cancers, although they may be more abundant in some subtypes (Anderson et al., 1994; Perrone et al., 2006; Sheng et al., 1990). Speculatively, the lack of selection for mutation may reflect the fact that the multiple RTKs that are overexpressed or mutationally activated in head and neck cancers are effective at keeping Ras at a high activity level in the absence of secondary mutation. Regardless, therapies that target important Ras-dependent effector pathways may have value in EGFR-dependent tumors, based on inactivation of the Raf-MEK-ERK effector arm. As one example, inhibition of KSR1, a kinase with scaffolding activity that promotes signaling between RAF, MEK, and ERK, was recently shown to sensitize EGFR and Ras-dependent tumors to ionizing radiation (Xiao et al., 2010).

4.1.4. STATs—The STAT (signal transducers and activation of transcription) proteins bind phosphorylated Y⁸⁴⁵ on EGFR, and are subsequently themselves phosphorylated by SRC and EGFR (Figure 4B). There are multiple members of the STAT family, with changes in the function of STAT1, STAT3, STAT5a and STAT5b known to contribute to the development of human cancer (Quesnelle et al., 2007; Lai and Johnson, 2010). The phosphorylated STAT protein then translocates directly to the cell nucleus, and activates the transcription of genes that support cell transformation, including iNOS (STAT3, (Lo et al., 2005)) and AURKA (STAT5, (Hung et al., 2008)). Head and neck cancers typically have hyperactive or overexpressed STAT3, linked to enhanced transcription of CCND1 (Masuda et al., 2002). STAT3 phosphorylation is also increased in head and neck cancers with poor prognosis, and increased STAT3 levels are associated with nodal metastasis (Arany et al., 2003; Leeman et al., 2006) in some studies, although at least one group did not identify any prognostic value of STAT3 used as an independent factor (Seethala et al., 2008), and one found a better prognosis (Pectasides et al., 2010). STATs have attracted interest as therapeutic targets in head and neck and other cancers. However, STATs are not catalytic, making the development of inhibitors relatively problematic. Attempts to disrupt the phosphorylation, dimerization, and DNA binding activity of these proteins, or to deplete STATs using oligonucleotides have not yielded a viable clinical candidate (Yue and Turkson, 2009). Although there is no doubt of the importance of this signaling effector in the EGFR cascade, it does not immediately offer a promising avenue for therapeutic development.

4.2. ErbB ligand-induced activation and extracellular modification of EGFR

In normal cells, EGFR is activated by the binding of ligands to the extracellular domain of the protein, resulting in conformational changes that activate the kinase activity. These ligands are typically produced by the cleavage of transmembrane precursor proteins, with the cleavage releasing soluble ~50–85 amino acid peptides into the extracellular environment. These ligands operate in three well-established modes (autocrine, paracrine, and juxtacrine); recently, a fourth mode of production, through exosomal release, was identified for at least some cancer types (Higginbotham et al., 2011), and is probably relevant to head and neck cancer. For EGFR, the most important ligands include EGF, betacellulin (BTC), epiregulin, transforming growth factor alpha (TGF- α), amphiregulin (AREG), and heparin-binding, EGF-like growth factor (HB-EGF). The cleavage of these proteins is performed by proteases of “a disintegrin and metalloprotease”, or ADAM, group, which are sometimes referred to as sheddases (Figure 4B).

In head and neck cancer, as in other cancers, both elevated expression of the ligands themselves and enhanced expression of the ADAM sheddases, have been shown to contribute to disease pathology and resistance to therapy. For example, increased epiregulin and amphiregulin expression was found in oral squamous cell cancers; high levels of epiregulin were associated with reduced survival (Shigeishi et al., 2008). Betacellulin is commonly expressed in head and neck cancer cell lines, supporting the EGFR-dependent activation of PI3K and MEK/ERK signaling (O-charoenrat et al., 2004). HB-EGF has been reported as abundant in head and neck cancer, with overexpression of HB-EGF induced in part by reduced expression of its negative regulator miR-212. Interestingly, the elevation of HB-EGF specifically was observed following treatment of patients with cetuximab, and was associated with acquired cetuximab resistance (Hatakeyama et al., 2010). In contrast, a study examining a panel of head and neck cancer cell lines identified lower expression of TGF- α and AREG associated with resistance to the EGFR-targeting agent gefitinib (Hickinson et al., 2009); as resistance also correlated with genomic gain or mutation of EGFR, the lower expression of activating ligands may reflect the ligand independence of these resistant lines.

Among the sheddases, increased activation of TACE (TNF- α converting enzyme, also known as ADAM-17) has been shown to elevate amphiregulin levels in head and neck cancer (Zhang et al., 2006). Several recent studies indicated TACE levels were significantly upregulated in head and neck cancer cell lines and primary tissue versus normal head and neck tissue (Ge et al., 2009; Kornfeld et al., 2011); one has found that TACE activity varied separately from total TACE expression, and was more associated with an aggressive tumor (Ge et al., 2009). Activation of TACE arises in part from phosphorylation by PDK1, which in turn is activated downstream of SRC and PI3K, connecting TACE activity to a feed-forward EGFR activation circuit, as well as making its activity to other stimuli connecting to SRC and PI3K (Zhang et al., 2006). Chemotherapy can induce TACE in at least some cancers, with activated Ras supporting this process (Kyula et al., 2010; Van Schaeybroeck et al., 2010), contributing to resistance to EGFR-targeting therapies. ADAM10 and a number of other ADAMs (Ko et al., 2007; Stokes et al., 2010), are also associated with head and neck cancer. Besides their action in the context of EGFR signaling, these ADAMs also target other proteins on the tumor cell surface, including cadherins and selectins, with cleavage of these targets contributing to tumor cell invasion. Drugs targeting ADAMs have been developed, and are progressing through clinical development ((Zhou et al., 2006); recently reviewed in (Saftig and Reiss, 2011). At present, these approaches have not achieved notable success, with first-generation trials halted due to adverse effects that may or may not be directly related to inhibition of the intended drug targets.

Although most discussions of post-translational modification of EGFR and other ErbB family members focuses on phosphorylation of the cytoplasmic domain, one class of extracellular modification, glycosylation, strongly influences receptor activation (Lane et al., 1985) and efficiency of antibody-based therapies. Multiple N-linked glycosylation events within domain III are essential for the conformational changes that occur following the binding of the EGF ligand: in the absence of such glycosylation, dimerization does not occur, reducing subsequent kinase activation (Fernandes et al., 2001). Specific glycosylations influence whether EGFR is in a high or low affinity binding state (Whitson et al., 2005). Use of tunicamycin to block N-linked glycosylation not only inhibits dimerization of EGFR, but also is active against the EGFRvIII protein, suggesting a potential clinical application (Fernandes et al., 2001). In fact, simultaneous treatment with tunicamycin makes some EGFR-dependent cancers more sensitive to erlotinib (Ling et al., 2009). Further, tunicamycin treatment resulted in a decrease in the steady-state levels not only of EGFR but also other ErbB family members and IGF1R, based on disruption of intracellular trafficking of these proteins. Moreover, cells treated with tunicamycin showed decreased survival signaling through AKT, and were markedly sensitized to radiotherapy (Contessa et al.,

2008). Finally, glycosylation also influences therapeutic response, influencing the binding of antibodies to EGFR by regulating epitope availability, or in some cases by contributing an epitope (Johns et al., 2005; Li et al., 2008).

4.3. EGFR signal inhibition, trafficking and stability

In addition to the pro-proliferation and survival proteins engaged by activated EGFR, additional proteins are recruited that serve as negative feedback controls. These fall into two main categories: attenuators of EGFR-dependent signals, or promoters of EGFR internalization and destruction. Treatments that enhance the activity of these feedback controls may provide useful therapeutic gains.

4.3.1. Signal attenuation—The protein tyrosine phosphatase SHP1 binds to EGFR-Y¹¹⁷³, as a later event (~30 minutes) after EGF stimulation of the receptor, following earlier (<15 minutes-post stimulation) binding of the proteins SHC, GRB2, and SOS. SHP1 binding attenuates EGFR signaling through the MEK/ERK effector pathway, dephosphorylating SOS. Adding an unexpected complication to this regulation, a recent study has found that EGFR is subject to methylation on R¹¹⁷⁵ by the arginine methyltransferase PRMT5, with methylated R¹¹⁷⁵ promoting Y¹¹⁷³ phosphorylation, and suppressing EGFR-dependent cell proliferation, migration, and invasion (Hsu et al., 2011). PRMT5-dependent methylation of EGFR is not EGF-responsive, but rather induced by interaction with cytoplasmic methylosome protein 50 (MEP50), expression of which has been shown in a breast cancer model to negatively correlate with disease state ((Peng et al., 2009); and discussed in (Hsu et al., 2011)).

4.3.2. Internalization and destruction—The E3 ubiquitin ligase CBL binds to EGFR-Y¹⁰⁴⁵, promoting internalization, ubiquitination and degradation of the protein (Oksvold et al., 2003). Specific relevance of this EGF-stimulated destruction pathway in response to DNA damage in head and neck cancer was recently demonstrated (Ahsan et al., 2010). Importantly, this study emphasized that order of treatment with EGFR-inhibiting agents and DNA damaging agents might be critical for the success of clinical strategies, as prior inhibition of EGFR antagonized subsequent EGFR internalization and destruction triggered by cisplatin and other DNA-damaging treatments. Cbl-dependent ubiquitination and internalization of EGFR also require activation-associated phosphorylation of EGFR on S¹⁰⁴⁶, S¹⁰⁴⁷, S¹⁰⁵⁷, and S¹¹⁴² (Oksvold et al., 2003, 2004) by Ca²⁺/calmodulin-dependent kinase II (Feinmesser et al., 1999), as well as on S⁹⁹¹, S¹⁰³⁹, and T¹⁰⁴¹ (Tong et al., 2009)). S¹⁰³⁹ and T¹⁰⁴¹ phosphorylation is conferred by the p38 stress-induced kinase, and treatment of cells with the selective p38 inhibitor SB-202190 blocks these phosphorylations (Tong et al., 2009).

Mechanistically, CBL modification of EGFR occurs at the plasma membrane, and promotes internalization in part by clathrin-mediated endocytosis (CME) (Tong et al., 2009). However, several studies have suggested that much of the EGFR internalized by CME remains active in signaling and is ultimately recycled to the cell surface (Goh et al., 2010; Sigismund et al., 2008; Sorkin and Goh, 2009). Recently, a number of studies have emphasized the importance of additional pathways for EGFR internalization, such as “non-clathrin endocytosis” (NCE) (Orth et al., 2006; Sigismund et al., 2008; Sigismund et al., 2005b). In contrast to CME, EGFR internalized by NCE is primarily shunted to the lysosome for degradation, making this an important route for downregulation of EGFR-dependent signaling. NCE is strongly inhibited by the cholesterol pathway inhibitory drug filipin, and some other cholesterol pathway-inhibiting drugs (Sigismund et al., 2008). This might suggest potential disadvantages of combining cholesterol inhibitors with EGFR-targeting agents that act by downregulating EGFR (for instance, monoclonal antibodies).

However, cholesterol pathway inhibition by lovastatin has been reported to improve the efficacy of gefitinib in NSCLC and glioblastoma (Cemeus et al., 2008; Park et al., 2010). There is some evidence that alternative pathways for EGFR internalization and downregulation are relevant to the action of receptor-targeting antibodies, such as cetuximab (Jaramillo et al., 2006). Clearly, more study of the underlying trafficking machinery is required.

In the past year, EGF-induced interactions between CBL, the GTPase dynamin 2 (DYN2), and a CBL-interacting scaffold protein, CIN85, were identified as necessary for EGFR movement from Rab7-positive late endosomes to sites of degradation (Schroeder et al., 2010). EGFR mutants spontaneously arising in NSCLC that have lost interaction with CIN85 are resistant to ligand-induced receptor downregulation (Yang et al., 2006). Through CIN85, CBL and EGFR bridge to multiple regulators of the internalization complex, including SRC kinases, which may be points of therapeutic modulation, e.g. by SRC inhibitors such as dasatinib. Binding HSP90 protects EGFR from interactions with CBL that lead to downregulation; inhibitors of HSP90 such as geldanamycin promote CBL-mediated loss of EGFR. However, the particular value of CIN85 itself as a biomarker or target at present is complicated (Yang et al., 2006). For example, high levels of CIN85 are associated with late-stage SCCHN and support signaling relevant to tumor proliferation (Wakasaki et al., 2010), opposite to expectations. Further clinical investigation is merited.

4.4. Nuclear EGFR

A nuclear fraction of EGFR is present in some head and neck cancers, with the likelihood of detecting nuclear EGFR increasing with increasing total EGFR content (Psyrry et al., 2008). Clinically, strong nuclear EGFR signal is associated with an aggressive tumor with poor prognosis for head and neck and other cancer types (Psyrry et al., 2008; Psyrry et al., 2005). In patients treated with radiation for locally advanced disease, nuclear localization of EGFR is associated with a higher risk of relapse and death (Psyrry et al., 2008). In the nucleus, EGFR has been reported to act as a transcription factor for cyclin D1 and other pro-oncogenic factors, and to phosphorylate targets such as proliferating cell nuclear antigen (PCNA), inducing cell growth and resistance to DNA-damaging treatments (reviewed recently in (Lo, 2010)). Mechanistically, the process for nuclear transport of EGFR has been described as involving action of SEC61 translocon and components of the endosomal transport machinery (Giri et al., 2005; Liao and Carpenter, 2007, 2009). In an NSCLC model, high-level expression of the ErbB ligands including EGF, amphiregulin, and others, and action of the SRC family kinases were found to promote nuclear EGFR expression; further, induced expression of nuclear EGFR promoted resistance to EGFR-targeting agents such as cetuximab (Li et al., 2009). A persistent question that has not yet been resolved is how an intact EGFR molecule, possessing a trans-membrane domain, is removed from the lipid bilayer, and operates in the nucleoplasm, even though a nuclear localization sequence has been suggested (Hsu and Hung, 2007). This point merits substantial further research effort. However, at present a significant body of evidence suggests that nuclear EGFR contributes substantially to the pathogenesis of EGFR-dependent cancers, serving as both a biomarker and potential treatment target.

5. Conclusions and Future Perspectives: moving towards a systems level approach to targeting SCCHN

Our understanding of the regulation of EGFR signaling has become bewilderingly complex. EGFR can be regulated by copy number, mutation, splicing, phosphorylation, ligand availability, dimerization partner availability, trafficking and degradation. EGFR output can be affected by changes in the expression or activation of its signaling effectors, or the

upregulation of other transmembrane receptors that compensate for inhibition of EGFR. In any given SCCN tumor, the relevance of each of these control mechanisms is largely unknown. In the past, biomarker studies have typically assessed one or two proteins for expression, mutation, or activation to find predictive correlates of treatment response. While some biomarkers are robust, as with KRAS mutation in colorectal cancer, the effect of most is more subtle. Multiplexed assays and efforts to assess signatures of treatment-responsive versus treatment-refractory tumors offer a fuller view, but do not capture the complexity of the signaling changes in tumors, whether before or following treatment. One exploratory goal in prognostic medicine is to overlay multiple high-throughput technologies, such as genomic sequencing, microarrays, phosphoproteomics, to gain a fuller understanding of the critical biological pathways in any individual tumor, to predict the best strategy for any patient in a personalized way (Friend and Ideker, 2011). Given the technical difficulties of some of the analyses involved, not to mention the computational difficulties of integrating and interpreting large orthogonal datasets, evaluation of the success of this approach likely lies some years down the road, as discussed in (Kohane and Margulies, 2011).

Although recognition of the complexity of biological networks has on some levels made it more difficult to identify appropriate therapeutic choices, on another level, insights from systems biology suggest a new way of thinking about treatment resistance that may directly lead to new designs for trials. In this view, it is recognized that cellular signaling networks have evolved to be robust, so as to make it possible to route around points of damage (Barabasi et al., 2011). While such robustness is beneficial to an organism in compensating for deleterious mutations, or in allowing organisms to survive under changing environmental conditions, a negative consequence of network robustness is in making it possible for tumor cells to route around the inhibition of oncogenes or their key effectors. In a robust network, it is necessary to develop a strategy that makes it impossible to route around a block, either by eliminating an essential, non-redundant central component, or alternatively, by simultaneously targeting multiple components that are able to compensate for each others activity.

To provide an example, in EGFR/ErbB signaling, SRC and related kinases have begun to be exploited as targets of interest. Although rarely mutated, SRC is often activated in solid tumors (Wheeler et al., 2009a). Active SRC contributes to EGFR signaling by placing key phosphorylations on EGFR, as discussed above. However, SRC also functions in multiple other signaling pathways, including notably the integrin-dependent cell adhesions/cell survival axis (Cabodi et al., 2010). Recent studies have documented that loss of responsiveness to ErbB-targeting agents such as trastuzumab is associated by activation of SRC, which compensates for loss of the upstream RTK (Zhang et al., 2011). Dual inhibition of SRC with EGFR (Wheeler et al., 2009b) or other ErbB proteins (Zhang et al., 2011), or EGFR effectors (Nozawa et al., 2008) predict that this strategy may have value in improving efficacy of these agents used alone.

Evidence for the role of SRC signaling in head and neck cancer, as well as the potential that SRC mediates resistance to EGFR inhibitors, have prompted the investigation of SRC inhibition in head and neck cancer. Preclinical studies indicate that dasatinib suppresses invasion and induces growth arrest and apoptosis in Tu167 head and neck squamous cell carcinoma cell lines (Johnson et al., 2005). Anti-invasive effects have also been demonstrated with saracatinib (AZD0530), an anilinoquinazoline SRC kinase inhibitor, which decreased oral squamous cell carcinoma invasion in Boyden chambers and in an orthotopic tongue cancer model, and reduced expression of the invadopodia markers cortactin, filamentous actin and phosphotyrosine (Ammer et al., 2009). Both agents have undergone phase II testing as single agents in head and neck cancer. The phase II trial of dasatinib enrolled 15 patients with recurrent or metastatic disease who had received at least

one systemic therapy regimen previously. No objective responses were observed and only two patients had stable disease at eight weeks. The median PFS was 0.9 months and median survival six months. Toxicity included pleural effusions, vomiting, and resulted in hospitalization, and toxicity was the reason for treatment discontinuation in four patients (Brooks et al.). Pharmacokinetic sampling in three patients who received dasatinib by percutaneous gastrostomy feeding tube revealed higher levels and faster elimination half-lives than predicted from the phase I data. A phase II trial of saracatinib monotherapy enrolled 9 patients with recurrent or metastatic disease, of whom 6 had received a prior chemotherapy regimen. In this trial, all patients had radiographic progression or clinical decline within the first 8 weeks, and the study was halted according to its early stopping rule (Fury et al., 2011). Thus, SRC inhibitors have not demonstrated clinical monotherapy activity in head and neck cancer. As of 2011, the question of whether SRC kinase inhibition can enhance the activity of EGFR inhibitors remains, and a phase I trial is currently ongoing to establish the safe dose of dasatinib which can be combined with cetuximab and radiation, with or without cisplatin (Egloff and Grandis, 2009; Gillison et al., 2008) [<http://clinicaltrials.gov/NCT00882583>].

Moving further afield, a recent siRNA library screen intended to identify genes that regulate sensitivity to EGFR inhibitors separately identified NEDD9, BCAR1, and SH2D3C as hits that are potent regulators in multiple cell types, including head and neck cancer (Astsaturon et al., 2010). Suggestively, each of these genes encodes a scaffolding protein that binds and regulates the activity of SRC and FAK in integrin-dependent pro-invasive and survival signaling, while NEDD9 and BCAR1 also connect directly to the EGFR effector SHC (Tikhmyanova et al., 2010) (Figure 5). NEDD9 also interacts directly with another known oncogenic kinase, Aurora-A (Pugacheva and Golemis, 2006). Astsaturon et al. went on to demonstrate that combining Aurora kinase inhibitors with EGFR inhibitors potentially reduced tumor cell growth both *in vitro* and in xenograft analysis, and showed that this was accompanied by general reduction in SRC kinase activity (Astsaturon et al., 2010). Further extending analysis of this network, Ratushny et al have recently found that dual inhibition of Aurora and SRC kinases is effective in reducing the growth of multiple classes of tumor cell lines (Ratushny et al., 2011). Cumulatively, this work is compatible with the idea that disruption of multiple proteins existing in a network proximally anchored to EGFR may have effectiveness, and suggests concepts that could support the development of multiple Phase I trials. It is likely that further probing of the network space around EGFR and its effectors, through direct functional assessments and screening, will suggest many more.

In summary, as of 2011, there are many targeted agents that are in, or close to, clinical trial for treatment of head and neck cancers. While most standard trial designs are likely to include a combination of targeted agents with cytotoxic drugs or chemotherapy, experience to date suggests that also combining multiple targeted agents in more complex therapeutic mixtures may be worth exploring. We are currently on a cusp with biomarkers, and transitioning from small datasets to large ones. The first genomic level sequences of HNSCC have just been published, with the promise of yielding many new functional insights into tumor pathogenesis (Agrawal et al., 2011; Stransky et al., 2011): for example, the unexpected finding of a high frequency of mutations in previously unlinked growth regulatory genes such as NOTCH1, IRF6, and TP63. At present, while it is likely that the criteria for selection of patients for trials will evolve significantly in the next several years, there is the need to accumulate and analyze some very large datasets to identify optimal approaches for personalized medicine. It will be an interesting decade.

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Figure 1. Approaches to engineer bispecific antibodies

The modular nature of the human IgG (A) serves as the basis for engineering a variety of bsAbs. Where appropriate clinical candidates have been identified along with stage of clinical testing. B) Heterodimeric Fc. “Knobs-into-holes,” a strategy developed by Carter and colleagues (Merchant et al., 1998), uses compensatory mutations in the CH3 domain of the Fc region to favor heterodimer formation. Additional strategies that improve upon heterodimer yield and stability have recently been described. These include more comprehensive compensatory mutations (Gunasekaran et al., 2010), SEEDbodies that take advantage of chimeric CH3 domains comprised of alternating segments of IgG and IgA sequence (Davis et al., 2010), and heterodimeric rat/mouse Fc domains (Jager et al., 2009). C) Dual-specificity Fv. Fv domains capable of binding with high affinity to more than one target (Bostrom et al., 2009). D) Insertion of additional variable domains. Structures such as the Di-diabody (Lu et al., 2005), DVD-IgG (Wu et al., 2009) and IgG-scFv (Orcutt et al., 2010) rely on insertion of additional variable domains into the basic IgG format at different locations E.) Bispecific-scFv molecules. Structures that take advantage of the antigen binding properties of the scFv.

2A

Figure 2. Structural consequences of EGFR mutations

A. Superposition of EGFR tyrosine kinase domain with C-SRC kinase domain. The sequence ELREA (746–750) deleted from EGFR in some head and neck cancers is shown in magenta. C-SRC has a three-residue deletion relative to wildtype EGFR in the same region, shown in orange. It loses one turn of the helix, as the ELREA mutation would be expected to do. The ATP analogue, phosphoaminophosphonic acid adenylate ester is shown in stick figure. The EGFR structure is PDB entry 2ITX (Yun et al., 2007) and the C-SRC structure is PDB entry 1K9A (Ogawa et al., 2002). **B.** Mutations in EGFR tyrosine kinase domain identified in HNC tumor samples. L858R is a common activating mutation in lung cancer. V765G is located on the C-helix of the N-terminal domain. K745R immediately precedes

the sequence ELREA deleted in some HNC. K745 hydrogen bonds to the alpha phosphate of ATP. The structure is taken from PDB entry 2ITX.

Figure 3. Homodimer of EGFR extracellular domains I-III

The variant III (EGFRvIII) deletion is colored magenta in the monomer on the left. EGF is in gray bound to both EGFR monomers. Cetuximab is in yellow. Cetuximab binding is not consistent with EGF binding or dimerization due to overlap of contacts with domain III. EGFRvIII is not likely to dimerize due to the absence of the tether loop in domain II which reaches to contact domain II of the other monomer. The EGF-bound EGFR homodimer is taken from PDB entry 1IVO (Ogiso et al., 2002) and the EGFR/cetuximab interaction is taken from PDB entry 1YY9 (Li et al., 2005b).

Figure 4. EGFR signaling interactions

A. Signaling downstream of EGFR family members, IGF1R and TGFβR. **B.** EGFR and c-MET downstream effectors. **C.** PI3K and PLCγ signaling downstream of EGFR. Violet, transcription factors; light blue, kinases; green, scaffolding proteins. Phosphorylation and oligomerization is only shown for EGFR/EGFR family receptors; see text for details. See for color reproduction the online version of this paper.

Figure 5. Signalling network integrated via scaffolding proteins NEDD9, BCAR1, and SH2D3C. See text for details. See for color reproduction the online version of this paper.

Table 1

EGFR-targeting agents in the clinic or under development

Monoclonal Antibodies			
Agent	Targets	Composition	Stage of Clinical Development for SCCHN
Cetuximab	extracellular epitope on EGFR ligand binding domain III	65% human, 35% murine IgG1 framework	Approved for use in SCCHN
Panitumumab	EGFR domain III	fully human IgG2	Approved for colorectal cancer, Phase I, II trials in SCCHN
Zalatumumab	EGFR domain III	fully human IgG1	Phase III
Nimotuzumab	EGFR domain III	humanized IgG1	Phase I, II
Necitumumab	EGFR domain III	fully human IgG1	pre-clinical
MEHD7945A	EGFR, ErbB3	IgG bispecific ab	Phase I
Small Molecule Inhibitors			
Gefitinib	ATP binding site of intracellular tyrosine kinase domain of EGFR	quinazoline-derived synthetic molecule	Phase II, III
Erlotinib	ATP binding site of intracellular tyrosine kinase domain of EGFR	quinazoline-derived synthetic molecule	Phase II
Lapatinib	ATP binding site of EGFR, ErbB2	quinazoline	Phase II, III
Afatinib	EGFR, ErbB2	anilino-quinazoline derivative	Phase II, III
CUDC-101	EGFR, ErbB2, HDAC	synthetic small molecule inhibitor	Phase I