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Targeting EGFR resistance networks in Head and Neck Cancer

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Abstract

A core set of oncoproteins is over-expressed or functionally activated in many types of cancer, and members of this group have attracted significant interest as subjects for development of targeted therapeutics. For some oncoproteins such as EGFR/ErbB1, both small molecule and antibody agents have been developed and applied in the clinic for over a decade. Analysis of clinical outcomes has revealed an initially unexpected complexity in the response of patients to these agents. Diverse factors, including developmental lineage of the tumor progenitor cell, co-mutation or epigenetic modulation of genes encoding proteins in an extended EGFR signaling network or regulating core survival responses in individual tumors, and environmental factors including inflammatory agents and viral infection, all have been identified as modulating response to treatment with EGFR-targeted drugs. Second and third generation therapeutic strategies increasingly incorporate knowledge of cancer type-specific signaling environments, in a more personalized treatment approach. This review takes squamous cell carcinoma of the head and neck (SCCHN) as a specific example of an EGFR-involved cancer with idiosyncratic biological features that influence design of treatment modalities, with particular emphasis on commonalities and differences with other cancer types.

Keywords

EGFR; epidermal growth factor receptor; head and neck cancer; signaling; resistance pathways; SCCHN

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1. Overview of Epidermal Growth Factor Receptor (EGFR) Signaling in Head and Neck Squamous Cell Cancer

1.1. EGFR and cancer

The 170 kD Epidermal Growth Factor Receptor (EGFR, also known as ErbB1), is one of four members of the ErbB/HER family of transmembrane tyrosine kinase growth factor receptors (Figure 1). EGFR forms dimers and higher order oligomers with itself and other members of the ErbB family (ErbB2/HER2/neu, ErbB3/HER3, and ErbB4/HER4) via a primary dimerization domain and several secondary receptor–receptor contact points [1-3]. Binding of naturally occurring extracellular ligands (e.g., amphiregulin, epiregulin, HB-EGF) to the extracellular ligand-binding domain (domain III) of EGFR induces conformational shifts that permit homo- and hetero-dimerization events between EGFR molecules and its family members. Dimer and multimer formation promotes tyrosine autophosphorylation of the EGFR intracellular domain; the resultant open configuration of the kinase domain enhances access by ATP and substrate and creates binding sites for signaling molecules. The EGFR kinase is also active at a low level when the protein is in the unliganded state, with the degree of activity varying by cell type, and influenced by both cell-intrinsic and environmental factors [4].

EGFR autophosphorylation activates multiple key signal transduction cascades that are mitogenic, antiapoptotic, angiogenic and pro-invasive (Figure 2). Ligand-bound receptor multimers are also internalized in clathrin-coated pits and targeted for ubiquitylation and destruction, or recycled to the cell membrane. Ultimately, EGFR kinase activity is restricted by the action of phosphatases. In cancer, as discussed below, it is common for EGFR activity to be greatly increased as a result of upregulation; amplification; mutation; or increased expression or activity of its activating ligands or cofactors. In addition, recent work has demonstrated the presence of phosphorylated EGFR in the nucleus of several tumor types, and the action of this nuclear EGFR as a transcription factor for proteins such as cyclin D [5,6]. Together, these increased EGFR signaling outputs drive the malignant behavior of human cancers. Inhibition of EGFR has been exploited or is under investigation as a therapeutic strategy in several solid tumor types, including colorectal cancer (CRC) [7-9], non-small cell lung cancer (NSCLC) [10], squamous cell carcinomas of head and neck (SCCHN) [11,12] and pancreatic adenocarcinoma [13]. Use of anti-EGFR antibodies (e.g. cetuximab [8]) and tyrosine kinase inhibitors (TKIs) (e.g. erlotinib) as single agents prolongs survival in metastatic colon and lung cancers, respectively. Inhibition of the EGFR pathway with cetuximab shows synergistic activity with conventional cytotoxic treatment modalities such as radiation [12] and chemotherapy [7,14].

Although EGFR signalling plays a critical role in tumorigenesis in a variety of tissues of origin, agents targeting EGFR are variably effective in different cancers. This presumably reflects the developmental origins of different tissues, environmental factors and host genetic background. Cell lineages utilize combinatorial dependence on signaling networks during development, so it is not surprising that tumors arising from different tissues reflect these changes. Tissue- and cancer- specific differences in the degree of dependence on EGFR signaling, resistance pathways in parallel receptors and downstream pathways, and response to pharmacologic interventions have been identified and characterized in several human malignancies. Improving therapeutic strategies will require an increasingly sophisticated understanding of tissue- and cancer-specific pathway dependence on the molecular components of EGFR signalling. A goal of this review is to focus on squamous cell cancers of the head and neck, and outline how EGFR targeting properties in this less-studied tumor type in some ways resemble but other ways differ those other cancers.

1.2. Squamous cell carcinoma of the head and neck (SCCHN) as a unique EGFR-dependent cancer

The incidence of SCCHN is predicted to have been over 35,000 cases in the United States in the year 2008 [15]. SCCHN cancers include cancers affecting non-keratinizing squamous epithelium of the mouth, nasal passages, sinuses, larynx and pharynx. These tissues arise in the aerodigestive tract, which is embryologically derived from the branchial arches; the mucosa in which tumors arise is endodermal. Several lines of evidence have pointed to EGFR as an important therapeutic target in SCCHN. EGFR expression is elevated relative to expression on normal adjacent squamous mucosa in over 80% of invasive head and neck cancers (Figure 3.I) [16]. High EGFR expression is associated with increased EGFR copy number and translates to worse outcome [17,18]. The prevalence of increased EGFR gene copy number in SCCHN varies by report, but is approximately 25% by polysomy and 33% by gene amplification [18-20]. There is an even distribution of EGFR FISH-positivity in carcinomas arising from any anatomical location [18].

The development of SCCHN is multifactorial, with contributions from lifestyle factors, genetics and viral infection. In particular, tobacco and alcohol are risk factors for SCCHN. Mutations of *TP53*, the gene encoding the tumor suppressor protein p53, are among the most common genetic alterations in SCCHN and have been associated with decreased overall survival [21]. The human papilloma virus (HPV) has also been shown to be associated with SCCHN, particularly oropharyngeal tumors [22-25]. The HPV protein E6, a mediator of HPV oncogenesis in the oral cavity, binds and inactivates the tumor suppressor p53 [26], suggesting at least one important causative role for viral infection in cancer etiology. Patients with stage I or II SCCHN are often cured with radiation therapy (RT) or surgery alone. However, more than half of patients present with locoregionally advanced disease and then prognosis is limited, with contemporary chemoradiation trials resulting in median survival of 19.1 months [27]. Multimodality treatment combining surgery, RT and chemotherapy results in cured patients who suffer from permanent functional impairment in speech, swallowing, and taste. Patients with recurrent or metastatic disease have even shorter life expectancies, with recent randomized studies of platinum-based combination chemotherapy reporting median survivals of less than 9 months [28]. When the levels of EGFR receptor, or concomitantly of EGFR receptor and its ligand are increased, the odds of recurrence and death are significantly increased (local recurrence rate 70% for > median EGFR versus 48% for ≤ median EGFR at 5 years and overall survival of 20% v. 38% at 5 years), whether primary management is surgical with external beam radiation or with induction chemotherapy [29-31], both emphasizing the functional contribution of EGFR to the disease, and suggesting it as a target. As discussed below, responses to therapy targeting EGFR may be complicated because of interactions with other signalling components and genetic and epigenetic changes characterizing SCCHN tumors.

1.3. Clinical inhibitors of EGFR

EGFR inhibition can be achieved with monoclonal antibodies (mAbs) directed to the extracellular moiety of EGFR, or with small molecular inhibitors of the EGFR kinase domain (Figure 4b). The first experiments to identify a mAb effective against EGFR in human cancers utilized radiolabelled M225, a murine mAb [32, 33]. Due to development of neutralizing anti-EGFR antibodies in patients treated with M225, the original 225 clone was engineered to a human IgG1 scaffold, yielding the chimeric antibody C225 (cetuximab, Erbitux™, IMC-225). Mechanistically, cetuximab interacts with domain III of EGFR, partially occluding ligand binding and sterically inhibiting the conformational shift required for dimerization [34]. Antibody-bound EGFR receptor is internalized by an alternate clathrin-independent endocytic trafficking, which directs the antibody-bound EGFR to caveolin-associated endosomes, from which EGFR is subsequently targeted for proteolytic degradation in lysosomes [35, 36]. Depletion of antibody-bound EGFR from the cell surface may be the most important underlying

mechanism of cetuximab activity *in vivo* [37], and, interestingly, SCCHN cell lines selected for cetuximab resistance have often acquired an endocytosis deficiency [38]. Cetuximab treatment up-regulates expression of p27^{kip1}, arresting cells in G1 [39]. Reduced proliferation and induction of apoptosis have been demonstrated in cetuximab-treated vulvar carcinoma A431 xenografts [40]. M225 substantially enhanced the antitumor effects of cisplatin in established xenografts of EGFR-expressing tumor cells [41], and as discussed below, EGFR inhibitors are commonly used in conjunction with classic chemotherapeutic agents.

Cetuximab and other EGFR-targeting mAbs (e.g. matuzumab and zalutumumab) also induce antibody-dependent cellular cytotoxicity (ADCC), activating a cytolytic T cell response that helps kill tumor cells [41]. Different mAbs vary in their ability to induce ADCC (e.g. panitumumab, which is IgG2, is a weak inducer of ADCC except in certain Fc receptor gamma polymorphisms). ADCC occurs through the binding of the Fc domain of an immunoglobulin molecule with Fc receptors located on phagocytes and natural killer cells. Studies of allelic polymorphisms of the Fc receptor gamma I (CD16) have demonstrated that variants with higher stimulatory activity on innate immune cells are associated with better outcomes in patients treated with EGFR-targeting mAbs [42,43]. While this has not yet been demonstrated in SCCHN, polymorphisms in Fc receptor alleles have been associated with altered response to treatment with the monoclonal antibody rituximab in lymphoma [44,45].

Aminoquinazolines, which include erlotinib, gefitinib and lapatinib, directly inhibit kinase function of the EGFR receptor by binding the intracellular tyrosine kinase domain. Both mAbs and TKIs block the ability of EGFR to activate downstream signaling cascades. However, TKIs do not affect EGFR internalization to the same degree as the EGFR-targeting mAbs. TKIs also do not induce the ADCC responses observed with mAbs. Both classes of EGFR inhibitors, mAbs and TKIs, are in clinical use.

1.4. EGFR inhibition in the treatment of head and neck cancer

For SCCHN, the mAb cetuximab yields an objective clinical benefit in both metastatic [14, 46] and locoregionally limited tumors [12]. A large, multi-institutional, randomized study of cetuximab in patients with locally advanced disease, has found that cetuximab contributes significantly to disease control and survival when added to definitive radiotherapy (RT) compared to RT alone [47]. Cetuximab also improves survival in metastatic and recurrent head and neck cancer. A randomized trial with cetuximab was conducted for first-line therapy in patients with measurable metastatic or recurrent SCCHN, comparing cisplatin plus placebo with cisplatin plus cetuximab [14]. The objective response rate was significantly higher in the cetuximab arm, which was associated with a hazard ratio for progression of 0.78, but the study was inadequately powered for progression and survival. First-line use of cetuximab was also studied in a randomized trial utilizing 6 cycles of platinoid plus 5-fluorouracil; there was a significant improvement in overall survival (OS) from 7.4 months in the chemotherapy alone group to 10.1 months in the cetuximab plus chemotherapy group [46]. Cetuximab also has activity in previously treated patients. Phase II trials in both the US and Europe tested the addition of cetuximab to cisplatin in patients who were already refractory to platinum-based chemotherapy [48,49]. Cetuximab monotherapy in patients with recurrent/metastatic SCCHN who had progressed on platinum-based chemotherapy resulted in an objective response rate of 13% [50]. Monotherapy response rates have been 10% or less for aminoquinazolines, with no survival benefit with gefitinib when compared to second line conventional chemotherapy [51-54]

Although the activity of EGFR inhibitors in SCCHN patients is clear, clinical results are modest. This suggests that majority of patients are either refractory from the commencement of treatment, or rapidly acquire resistance. At present, there are few reliable indicators of which patients are most likely to respond well to cetuximab or other EGFR inhibitors, although work

in this critical area is ongoing [55]. Differences in outcome between antibodies and small molecules directed against EGFR may be the result of ADCC for mAbs, broader target specificity for TKIs, and differences in pharmacokinetic and pharmacodynamic properties of individual agents, and significant pharmacogenomic variation in TKI absorption and metabolism. Thus, the modest results of EGFR antagonists in SCCHN, despite the dependence of these cancers on EGFR signaling suggested by the frequent overexpression and other evidence discussed below, suggests the presence of acquired or intrinsic resistance pathways in head and neck cancer. Therapeutic resistance may be overcome and clinical outcomes improved via development of combination therapies that targets the resistance pathways, which increasingly appear to cluster in a network of signaling proteins that connect closely with EGFR (Figure 4c).

2. Known and Potential Modulators of EGFR Sensitivity in SCCHN

A number of molecular mechanisms have emerged as likely candidates to influence the responsiveness of individual SCCHNs to EGFR inhibitors. As outlined below, these mechanisms are likely subject to variation in different patients, and affect i) EGFR-ligand interactions (Figure 3); ii) EGFR co-receptor redundancy and utilization of alternative receptors (Figure 1); iii) expression and activity of EGFR signaling effectors (Figure 2).

2.1. Regulatory mechanisms operating at the level of the EGFR receptor and its ligands

2.1.1. EGFR expression levels—Initial biomarker studies in trials of EGFR inhibition in SCCHN addressed the idea that EGFR expression levels might themselves predict responsiveness. EGFR immunohistochemical staining intensity was examined as a predictor of outcome in an ECOG study of cisplatin with placebo or with cetuximab in metastatic/recurrent disease [14]. EGFR expression was dichotomized as very high staining intensity and density (those patients with 3+ staining on $\geq 80\%$ of cells), or low-moderate (all others). Patients with very high EGFR staining were less responsive to chemotherapy and derived no benefit from the addition of cetuximab, whereas the addition of cetuximab significantly improved response rate (from 12 to 40%, $p=0.02$) in those with low-moderate staining. These findings suggest a number of possibilities, including that current doses are inadequate when receptor content is high, or that ligand-independent activation (not amenable to inhibition by cetuximab) is more prevalent when receptor content is high. Another study analyzed paired tumor specimens and skin biopsies from patients, before and after treatment with erlotinib and cisplatin in a phase I study [56]. In an analysis of 9 paired tumor biopsy specimens and 32 paired skin biopsies, a reduction in phosphorylated EGFR in both skin and tumor after 7 days of erlotinib correlated with an increase in time to progression (TTP) and overall survival. Increased levels of EGFR expression as detected by immunohistochemistry are associated with inferior survival and increased locoregional failure [30,57,58]. These simple analyses are suggestive that EGFR levels predict for treatment response, but not sufficient to rigorously discriminate responders from non-responders.

Polymorphisms within the *EGFR* gene and promoter have been associated with increased expression of *EGFR* mRNA and protein [59] and resistance to treatment with EGFR inhibitors [60]. There are two common single nucleotide polymorphisms which exist in the *EGFR* promoter region; -216 G/T is located at the binding site for the SP1 transcription factor, and -191 C/A is located in close proximity to the transcription initiation site [61]. There was a significantly higher *EGFR* promoter activity in T-C versus G-C variants, with the G/T substitution contributing to a 30% increase in promoter activity. Electrophoretic mobility shift assay showed a significantly increased affinity of nuclear proteins and purified SP1 protein to the -216T allele in comparison to the -216G allele.

Germ-line polymorphisms in introns of the *EGFR* gene have also been implicated in intrinsic resistance to *EGFR* inhibitors in SCCHN. Such polymorphisms are known to exist in lung cancer [62,63]. The *EGFR* gene contains a highly polymorphic region in intron 1, with a variable number of CA single sequence repeats (CA-SSR) ranging from 9 to 21 [64]. The number of CA dinucleotides affects *EGFR* gene transcription; as head and neck cancer patient samples or cell lines with lower CA-SSRs express higher levels of *EGFR* mRNA and protein [65,66]. *EGFR* mRNA and protein levels were also inversely correlated with CA-SSR number in a panel of 12 SCCHN cell lines [60]. Cells with a lower number of CA-SSRs, and hence higher *EGFR* levels, were more sensitive to the growth inhibitory effects of erlotinib, and sensitivity could be reversed by siRNA-mediated depletion of *EGFR*. CA-SSR were identical in tumor and normal tissue samples from a group of 30 subjects with advanced SCCHN, confirming germ-line inheritance rather than selection during tumorigenesis. The facile measurement of CA-SSR number from DNA collected from normal skin or blood samples of patients with SCCHN may assist in predicting the sensitivity of patient tumors to *EGFR* inhibitors [60]. Interestingly, skin toxicity has been established as a good correlative marker of treatment response to *EGFR* inhibitors in SCCHN [14]. A lower number of CA-SSRs was associated with increased skin toxicity to *EGFR* inhibitors in a clinical trial of colorectal cancer patients [60]. Since SCCHN cell lines with lower CA-SSRs are more sensitive to the growth inhibitory effects of *EGFR* inhibitors, these findings may provide an explanation of why increased *EGFR*-inhibitor induced skin toxicity is associated with a favorable prognostic outcome in SCCHN.

Epigenetic modifications of *EGFR* have also been identified and may be pertinent to *EGFR* expression and response to *EGFR* inhibitors. *EGFR* contains large CpG islands that extend into exon 1. Cytosine methylation of these sites may lead to transcriptional silencing of *EGFR*. In one study, methylation of CpG islands of *EGFR* was detected in 6 of 17 (35%) SCCHN tumor samples as well as 3 of 15 (20%) breast tumors and 6 of 54 (11%) lung tumors [67]. Methylation was associated with transcriptional silencing of *EGFR* in CAMA1 and MB453 gefitinib-resistant breast cancer cell lines and treatment with decitabine, a DNA methyltransferase inhibitor, restored expression of *EGFR* and sensitivity to gefitinib. Histone acetylation is another epigenetic regulator of gene expression which may play a role in transcriptional regulation of *EGFR*. Treatment of *EGFR*-overexpressing estrogen MDA-MB-231 breast cancer cells with a histone deacetylase (HDAC) inhibitor resulted in decreased *EGFR* mRNA stability *EGFR* expression and mRNA stability [68].

2.1.2. Kinase domain mutations—Mutations in the kinase domain of EGF have a well-established role as modulators of *EGFR*-inhibitor response in patients with NSCLC and other cancers. These tumor intrinsic somatic mutations, typically comprising in-frame deletions and amino acid substitutions clustering around the ATP binding pocket of *EGFR*, increase access to both ATP, resulting in increased ligand-dependent activation, and the competitive kinase inhibitors, resulting in increased sensitivity of *EGFR* to inhibition by TKIs (Figure 3.II). The prevalence of tyrosine-kinase mutations in head and neck cancer is low. A screen of kinase domain mutations in the tumor DNA of 100 patients of Caucasian ethnicity with SCCHN yielded only one somatic *EGFR* missense mutation, K745R, in the ATP binding cleft of *EGFR* [69]. Another study failed to detect any *EGFR* kinase domain mutations in tumor specimens of 8 SCCHN patients who responded to *EGFR* TKI's [70], while a third recent study failed to detect any mutations in *EGFR* from a sample of 91 SCCHN from patients of Japanese origin [71]. However, 3 out of 41 (7.3%) tumors of SCCHN patients of Asian origin possessed kinase domain mutations in *EGFR* [72], with all three patients having the same mutation: an in-frame deletion in exon 19 (E746_A750del). Interestingly, Asian populations have been reported to have higher rates of *EGFR* kinase domain mutations in lung cancer (reviewed in [73]). The differences among these studies may be accounted for by the increased proportion of lung cancer patients who are nonsmokers in Asia compared to Western countries, suggesting a

possible role for tobacco smoke in creating a hyper-mutational environment in which mutation of EGFR itself is not strongly selected

2.1.3. EGFR variant III—Variant forms of EGFR have been identified in human cancers, and arise from either abnormal splicing or somatic deletions [74]. EGFR variant III (also known as EGFRvIII, ΔEGFR, del2-7EGFR and mEGFR) is the most frequently detected truncation mutant. It contains an 801 base pair in-frame deletion that results in the loss of 267 amino acids corresponding to exons 2 through 7 in the extracellular domain. The functional consequence of this mutation is to eliminate portions of the extracellular EGF-binding domain and the cysteine-rich domain, which promotes kinase-activating dimerization (Figure 3.III). EGFRvIII is a constitutively active form of the receptor that is only found in tumors [75-77]. In spite of the loss of extracellular domain II, EGFRvIII does not have an altered ability to undergo dimerization [78]. Although domain II clearly contributes to binding, contacts provided by other domains can compensate for its loss in EGFRvIII [79].

EGFRvIII is found in glioblastoma, breast, ovarian, prostate, and lung carcinoma [80-83], albeit sometimes at relatively low frequencies (e.g., 5% of squamous cell lung cancers, and not at all in lung adenocarcinomas [84]). However, EGFRvIII is the most common EGFR mutation in glioblastoma, present in 25-50% of cases [85,86]. About 40-50% of glioblastoma samples also have EGFR amplification, and no EGFRvIII mutations were identified in the tumors without EGFR amplification [87-90]. Amplification or increased expression of EGFR in SCCHN occurs in only about 10 percent of cases [77]. The EGFRvIII splicing variant is found as commonly in SCCHN as in glioblastoma, and is tumor intrinsic, as EGFRvIII is not detected in adjacent normal mucosa in SCCHN [91]. In one study, 42% of SCCHN tumors (n=33) evaluated expressed EGFRvIII, with all of the EGFRvIII-expressing tumors also expressing full length EGFR [91]. An additional truncated mutation of EGFR has been identified uniquely in both normal and malignant keratinocytes of the oral mucosa [92,93], although the exact function of this truncation mutant remains unknown.

Evidence from clinical trials in glioblastoma has examined the role of EGFRvIII as a predictive factor in cancer outcomes [94]. There is clinical evidence that EGFRvIII status can predict the response to erlotinib or gefitinib [95]. The expression of EGFRvIII was assessed by immunohistochemistry in a study of 649 patients with glioblastoma and looked at EGFRvIII status in addition to the traditional RTOG recursive partition analysis (RPA) prognostic factors. Activation of AKT/MAPK pathways were predictive of poor outcomes in EGFRvIII-negative patients but not EGFRvIII positive patients [96]. The presence of EGFRvIII does not seem to predict treatment outcomes to EGFR inhibition. Two phase II trials testing EGFR inhibitors in recurrent high-grade gliomas have not been successful in trials, even when examining subsets of patients with EGFR overexpression or EGFRvIII mutations [97,98].

The role of EGFRvIII signaling in SCCHN remains an area of active investigation. In glioblastoma cells, EGFRvIII has been implicated in radioresistance and alterations in proteasome composition [99]. Ecotopic expression of EGFRvIII in CHO cells and a dominant-negative EGFR in mammary carcinoma cells confirms that the constitutively active EGFRvIII confers a cytoprotective response and relative radioresistance [100,101]. SCCHN cells lines transfected with EGFRvIII had decreased levels of apoptosis when treated with cisplatin and decreased growth inhibition by cetuximab compared to control treatment [91].

The presence of EGFRvIII may also account for limitations in the responsiveness of tumors to EGFR inhibition, either with antibodies (e.g. C225) or TKIs (e.g. erlotinib, gefitinib). C225 can still bind to EGFRvIII, which is internalized in response to treatment in a system with EGFR wild-type (EGFRwt) expression [102]. Investigators testing the ability of EGFRvIII to internalize noticed significantly decreased and delayed internalization of variant III in a system

without EGFRwt [103-106]. Due to these functional results, the prevalence of EGFRvIII mutations in approximately 40 percent of SCCHN tumors, and the detection of variant EGFR in tumor but not normal tissue, EGFRvIII itself has been proposed as a therapeutic target in SCCHN. A variety of therapeutic approaches have been developed to target EGFRvIII, including immunotherapy/vaccination [104-106], small molecules [107] and molecular/antibody neutralization [108-111]; their pre-clinical evaluation is ongoing.

2.1.4. Glycosylation of EGFR—Glycosylation of EGFR has long been known to be important for both ligand binding activity and kinase activation of the receptor [112,113]. Twenty percent of the mass of extracellular EGFR results from attached carbohydrates, based on the presence of at least 9 active N-linked glycosylation sites on the extracellular domain of EGFR (Figure 3.IV) [114,115]. Of these, four sites within domain III (N328, N337, N389, and N419) have been identified as contributing to ligand-induced receptor activation [116]. In normal EGFR activation, domain III binds EGF ligand and subsequently undergoes a conformational change that is required for dimerization: this process is blocked in the absence of glycosylation. Inhibition of N-linked glycosylation by growth of cells in tunicamycin, an inhibitor of N-linked glycosylation, decreases the dimerization not only of EGFR, but of the EGFRvIII splice variant [116].

Glycosylation has important implication for therapeutic development: for both cetuximab and newer directed therapeutic antibodies, such as IMC-11F8, the antibody epitopes are constrained to recognize parts of the EGF-binding moiety that are not obscured by bulky carbohydrate groups, resulting in near-identical recognition sites on EGFR in spite of entirely different antibody-EGFR interface contacts [34]. In addition, tunicamycin treatment of cells reduces the cycling of EGFR to the cell surface, increasing retention of EGFR in the endoplasmic reticulum (ER): this is accompanied by a decrease in EGFR-dependent AKT activation, and radiosensitization of tumor cells in glioma and pancreatic cancer models [117]. Tunicamycin co-treatment also sensitizes tumor cells to treatment with treatment with erlotinib [118]. Whether these results are physiologically relevant to EGFR signaling and impact EGFR-directed therapies in SCCHN has not yet been examined, but it is likely these signaling relationships will pertain.

2.1.5. Nuclear EGFR—One of the more startling activities to be identified for EGFR is in the nucleus, as a transcription factor (Figure 3.VI). After a series of initial observations in the 1990s, a rigorous 2001 study used a combination of cell fractionation, immunofluorescence, and direct DNA binding and transcription assays to demonstrate the direct binding of EGFR to a motif within the promoter of cyclin D1 [6]. This binding, and transcriptional activation of cyclin D1, was directly regulated by EGF treatment of cells [6]. The mechanism of translocation of EGFR to the nucleus remains unclear. A putative nuclear localization sequence resides at amino acids 645-657 of the cytoplasmic domain, and fusion of this motif to beta-galactosidase translocates this protein to the nucleus [6]. At least one splice variant of EGFR has been identified that lacks transmembrane sequences, and this variant shown to localize to the nucleus in an EGF-dependent manner [119]. A significant number of receptor tyrosine kinases (RTKs) have now been identified as translocating to the nucleus under some growth conditions, with this translocation important for their biological action: overall, this area needs significantly more study to elucidate mechanisms of action. In the interim, nuclear EGFR has been implicated in additional direct roles in transcription, such as an interaction with STAT5 that induces the mitotic regulator and oncogene, Aurora-A [120]. These nuclear actions are potentially important for EGFR functions in cancer, as several studies have noted that nuclear EGFR is a significant prognostic factor for aggressive cancers of various types, including SCCHN cancers [5, 6]. However, high levels of nuclear EGFR tend to accompany higher levels of EGFR overall, and at this time, the specific importance of EGFR-dependent direct gene

regulation (aside from indirect signaling effects) is unknown. The presence of nuclear EGFR and its function in SCCHN remains to be fully described and characterized.

2.1.6. EGFR ligands and ADAM family sheddases—Transmembrane precursors of EGFR ligands are expressed on the cell surface, and must be proteolytically activated to release the active ligand fragments to the interstitial space (Figure 3.V). EGF, betacellulin, epiregulin, transforming growth factor alpha (TGF- α), amphiregulin, and heparin-binding, EGF-like growth factor (HB-EGF)[121] are activated by a family of proteases (sheddases) also known as “a disintegrin and metalloprotease”, or ADAM, proteases. Once released from the membrane, these 49-85 amino acid mature growth factors are able to bind EGFR and family members. Expression levels of the ligands and the sheddases has been linked to negative outcomes, both as correlate and contributing agent in several types of cancer [122-124]. In SCCHN, expression of amphiregulin predicts sensitivity to cetuximab and gefitinib [125]. Elevated expression of epiregulin is prognostic of shorter survival in oral SCC, and is tumor promoting in oral SCC cells [126, 127]. Activation of ADAM-17 (also known as TACE) by gastric releasing peptide (GRP) is a potent activating stimulus to increase amphiregulin levels and activate EGFR in SCCHN [128]. These and other studies [129] suggest the significant importance of this control mechanism for SCCHNs, along with other cancers. As a result, sheddases have attracted considerable attention in their own right as drug targets, with agents targeting ADAM-10 and ADAM-17 moving through the development process [130, 131].

2.2. Receptor redundancy and EGFR co-receptors

2.2.1. ErbB2, 3, and 4—As noted above, EGFR/ErbB1/Her-1 is one of four known members of an RTK family. The other family members, ErbB2/Her-2/Neu, ErbB3/Her-3 and ErbB4/Her-4, can either heterodimerize with EGFR and affect its signaling, or alternatively, dimerize amongst themselves, and compensate for inhibition of EGFR signaling. A number of excellent recent reviews have summarized the interactions among these proteins [132-136], with some specifically emphasizing their importance and action in SCCHN.

ErbB2 is a favored dimerization partner for the other ErbB family members [137]. ErbB2 has long been known to affect tumor aggressiveness and malignant potential in human malignancies, particularly breast cancer. In SCCHN, studies of ErbB2 suggest overexpression in ~ 30% of primary tumors, with slightly higher or lower numbers in different studies likely reflecting limitations of available antibodies [138,139]. ErbB2 expression levels have been associated with malignant potential in oral cavity SCC [140]. Some studies have suggested expression level of ErbB2 may predict sensitivity to cytotoxic agents including cisplatin and 5-fluorouracil in SCCHN [141]. Continued investigation of resistance factors may improve clinical practice involving ErbB2-targeting agents.

Several studies have examined SCCHN specimens for characteristic patterns of expression of ErbB3 and ErbB4. One study focusing on oral cavity SCC had a low rate of ErbB-3/4 detection by immunohistochemistry [142], but another investigation looking at SCCHN cell lines from a variety of sites shows ErbB3 in a substantial proportion of lines, while ErbB4 is rarely detected [143]. ErbB4 has been found in a greater percentage of newly derived SCCHN cell lines compared to established lines [144]. Importantly, expression levels of ErbB3 were associated with resistance to the TKI, gefitinib, but not to the anti-ErbB1 mAB, cetuximab [143]. Further investigation is required to determine the viability of ErbB3 and ErbB4 as therapeutic targets in SCCHN.

2.2.2. IGF and c-Met—Activation of alternative parallel signaling pathways has been shown to promote cancer cell survival under the selective pressure of EGFR inhibition (Figure 1). Insulin-like growth factor I receptor (IGF-IR) is a transmembrane RTK which is expressed in

many normal tissues and has been shown to play a role in epithelial cancer cell development [145,146]. Among other functions, IGF-IR transmits cellular survival and motility signals through insulin-receptor substrate-1 (IRS-1) which activates the PI3K>AKT pathways and transmits cellular proliferation signals through Shc which activates the Ras>Raf>MEK>ERK core growth pathway [145,147].

While its role in the pathogenesis of head and neck cancer has not been fully elucidated, IGF-IR was shown to be overexpressed in human SCCHN cell lines and tumors [146]. Phase I clinical trials are ongoing using an IGF-IR blocking antibody in SCCHN [148]. Importantly, IGF-IR was shown to play a role in EGFR resistance in cell lines representing multiple cancer types including breast, prostate [149] and NSCLC [150]. EGFR was detected in IGF-IR immunoprecipitates in mammary epithelial cells, and IGF-I treatment increased the amount of tyrosine-phosphorylated EGFR in these complexes [151]. Treatment with ZD1839, a specific EGFR inhibitor, prevented IGF-I induced activation of ERKs but not AKT. The authors suggest that IGF-IR transactivates EGFR through heterodimeric complex formation and trans-phosphorylation of EGFR by IGF-IR. In this function, IGF-IR may stimulate cell proliferation by activating the ERK pathway and thus inducing resistance to EGFR inhibitors [150,151]. This novel function of IGF-IR has been validated in NSCLC cell lines [150] and most recently in a SCCHN model [146]. Stimulation of head and neck cell lines with IGF resulted in heterodimerization of IGFIR with EGFR and caused activating phosphorylation of both receptors [146]. Furthermore, treatment with a combination anti-IGF-IR therapeutic antibody, A12, and cetuximab more effectively inhibited cellular proliferation and migration than treatment with the individual agents. The potential of this novel drug combination for the treatment of EGFR-resistant SCCHN in the preclinical and clinical settings remains to be elucidated.

The transmembrane receptor tyrosine kinase MET was shown to contribute to resistance to EGFR inhibitors in cell lines derived from head and neck, breast, gastric and lung cancers as well as lung tumor samples. In one study, MET amplification was detected in 9 of 43 (21%) lung tumors of patients who acquired resistance to EGFR inhibitors, but in only 2 of 62 (3%) of lung tumors with EGFR mutations from patients who were never treated with kinase inhibitors [152]. MET amplification causes gefitinib resistance by driving ERBB3-dependent phosphorylation, and maintaining persistent activation of the PI3K/Akt signaling in the presence of EGFR inhibition [153]. In the absence of MET amplification, MET signaling can be activated by increased expression of the MET ligand hepatocyte growth factor (HGF), leading to resistance to EGFR TKIs in lung adenocarcinoma patients harboring EGFR-activating mutations, again associated with persistent PI3K/AKT activation [154]. MET signaling also contributes to intrinsic resistance to EGFR tyrosine kinase inhibitors in breast cancer, where MET activates c-Src in a resistant breast cancer cell line, leading to tyrosine phosphorylation of EGFR even in the presence of EGFR TKIs [155]. Cortactin, a regulator of dynamic actin networks, was shown to regulate MET signaling and promote resistance to EGFR inhibitors in SCCHN cell lines [154]. When overexpressed in SCCHN cells, cortactin stabilized MET, enhanced HGF-mediated mitogenesis and activated Akt, leading to resistance to gefitinib. Interestingly, the gene encoding cortactin, *CTTN*, resides on chromosomal locus (11q13) that is frequently amplified in head and neck cancers.

2.2.3. VEGFR—Tumor-induced angiogenesis plays an important role in cancer development and has been linked to EGFR resistance. Activation of EGFR signaling by EGF results in secretion of proangiogenic growth factors such as vascular endothelial growth factor (VEGF); blockade of EGFR inhibits the secretion of VEGF. Vitoria-Petit and colleagues established A431 human squamous cell carcinoma cell lines resistant to EGFR inhibition after continuous treatment of xenograft tumors with anti-EGFR antibody [156]. Five of six of these resistant A431 cell lines expressed elevated levels of VEGF and displayed increased angiogenic

potential *in vitro* and tumor angiogenesis *in vivo*. Furthermore, A431 cells genetically engineered to overexpress VEGF displayed resistance to anti-EGFR antibodies *in vivo* [156]. Thus, resistance to EGFR antagonists may well be mediated via VEGF signaling.

Tumor-induced angiogenesis may be involved in the development and progression of SCCHN. A meta-analysis of 12 studies looking at VEGF protein overexpression and clinical outcome of patients diagnosed with SCCHN concluded that the overall risk of death in 2 years was approximately 1.9-fold higher in patients with VEGF-positive tumors (as detected by immunohistochemistry) [157]. The co-overexpression of VEGF and VEGF receptor 2 (VEGFR2) was correlated with a higher tumor proliferation rate and worse survival in patients with SCCHN [158]. The cross-talk of VEGFR and EGFR signaling cascades (Figure 1) in the biological context of SCCHN is not fully characterized; however, clinical studies are ongoing to explore the combination of anti-VEGF and anti-EGFR therapies in SCCHN.

2.2.4. Additional resistance factors—Additional examples of signaling cross-talk between EGFR and other environmental or growth factor receptors have been described in SCCHN, although the impact of these signaling interactions for pathway activation and resistance to therapy are not as well understood. Interactions with the extracellular matrix and involving lateral cell connections play a role in EGFR signaling. Normal squamous epithelium requires cell-to-cell contacts for survival. Apoptosis resulting from the disruption of these interactions is referred to as anoikis [159-161]. Malignant cells taken from oral SCCs have been shown to survive via E-cadherin mediated anoikis-resistance pathways that signal through EGFR [162]. EGFR signaling also can affect cell migration and the breakdown of extracellular matrix (ECM) components. The ability of cigarette smoke promote ECM breakdown and invasiveness via EGFR signaling has been described [163]. The downregulation of urokinase plasminogen activator receptor (uPAR) acts synergistically with EGFR inhibition in SCCHN cells [164]. Additionally, G-protein coupled receptors (GPCR) may be involved in cross-talk with EGFR [165]. The binding of ECM by integrins and signaling through integrin-linked kinase (ILK) affects cellular radiosensitivity and EGFR-dependent survival in SCCHN cells [166]. To date, these observations have not been exploited in design of therapeutic approaches.

2.3 Resistance mechanisms originating from EGFR downstream effectors

Figure 2 shows the major downstream signaling proteins that are known to interpret EGFR-initiating signals. Many studies have documented changes in this protein network as contributing to resistance to EGFR. The most important sources of resistance in SCCHN are outlined below.

2.3.1. PI3K / AKT / PTEN—Phosphoinositol-3-kinase (PI3K) is a cytosolic heterodimeric complex which comprises a regulatory p85 subunit (encoded by the PIK3R1 gene) and a catalytic p110 subunit (encoded by PIK3CA) [167]. EGFR activates PI3K by multiple routes, including PI3K binding to the phosphorylated IRS-1 adaptor coupled to active EGFR [168], direct PI3K binding to the EGFR heterodimerization partner ErbB3 [169], or based on initial activation of RAS, which binds and activates PI3K. Upon activation, PI3K catalyzes the formation of phosphatidylinositol 3,4,5-triphosphate (PIP₃), which acts a second messenger to recruit AKT to the cell membrane. Tumor-suppressor protein PTEN (phosphatase and tensin homolog) is a phosphatase that acts as a negative regulator of PI3K [167]. Enhanced activity of PI3K or decreased activity of PTEN activates AKT. AKT acts on downstream substrates that promote cell survival signals, and other such as mTOR and its target p70S6K that promote protein synthesis, and glycogen metabolism [148,170]. The tumor suppressing activity of PTEN is partially dependent on the induction of cell cycle arrest in the G1 phase of the cell cycle [171]. PTEN induces cell cycle arrest by upregulating the cyclin dependent kinase (CDK)

inhibitor protein p27Kip1 and downregulating the G1/S transition mediator, Cyclin D1 [172-174]. As discussed below, these and other core cell cycle regulators have been implicated in resistance to EGFR inhibitors in SCCHN.

Mutations in *PIK3CA*, encoding the catalytic subunit of PI3K, have been reported in SCCHN, particularly in pharyngeal cancer samples [175]. *PIK3CA* and *AKT2* gene amplification, AKT activation, and PTEN protein downregulation have also been reported in SCCHN [176-178]. In other cancers, mutations inactivating the negative regulatory *PIK3R1* subunit have been reported (e.g. [179]), as have mutation of alternative isoforms of the PI3K catalytic subunit *PIK3CB* [180-183], with some of the observed mutations clearly creating an oncogenic function [183]; the occurrence of such mutations in SCCHN has not yet been investigated.

Deregulation of the PI3K/AKT pathway is associated with resistance to EGFR inhibitors. Bianco and colleagues reported that while gefitinib treatment eliminated EGFR phosphorylation in both the EGFR-dependent A431 (gefitinib-sensitive) and MDA-468 (gefitinib-resistant) cancer cell lines, the basal activity of AKT was maintained in MDA-468 cells [184]. In contrast to A431 cells, MDA-468 cells lack PTEN; following introduction of PTEN into MDA-468 cells, gefitinib treatment reduced the activity of AKT, induced apoptosis and promoted cell cycle delay [184,185]. Loss of PTEN, or upregulation of AKT, also increases vascular endothelial growth factor (VEGF) production and activity of the HIF-1 transcription complex, promoting tumor growth in hypoxic microenvironments and increased angiogenesis (reviewed recently in [186]). The role of PTEN mutations in determining the EGFR inhibitor sensitivity of SCCHN tumors remains to be investigated.

2.3.2. Cell cycle regulators—In normal cells, transcription of *CCND1* (encoding cyclin D1) is regulated by EGFR-RAS-RAF-ERK dependent signaling cascades, and in transformed cells is influenced by expression of nuclear EGFR [6]. Cyclin D1 is overexpressed in up to 68% of SCCHN and is associated with local invasion and poor prognosis, [187-189]. Upregulation of Cyclin D1 has been specifically associated with resistance to gefitinib in SCCHN [190], in a study where three of six cell lines with *CCND1* gene amplification and cyclin D1 overexpression were also resistant to gefitinib treatment as assessed by clonogenic survival assays. This study also showed that exogenous overexpression of cyclin D1 in a gefitinib-sensitive, low *CCND1*-expressing cell line induced resistance to gefitinib-induced G1/S growth arrest.

The cyclin-dependent kinase (CDK) inhibitors p27Kip1 and p21Cip1/Waf1 are members of the CIP/KIP family, which inhibit G1/S transition by binding and blocking activation of the CDK2/cyclin E and CDK2/cyclin A complexes. EGFR-dependent signaling has been implicated in negatively regulating the transcription of p27Kip1 [191,192]. p27Kip1 was reported as downregulated in 44% of SCCHN tumors [193], and as upregulated in 59% of skin biopsies following treatment of advanced SCCHN patients with erlotinib [194]. Stimulation of the activity of p27Kip1 and p21Cip1/Waf1 has been associated with the anti-proliferative effects of gefitinib in one study performed in SCCHN cell lines [195]. Di Gennaro and colleagues demonstrated that the gefitinib-induced G1/S arrest in SCCHN cell lines was associated with an increased expression of both p27Kip1 and p21Cip1/Waf1, and reduced in cells transfected with p27 and p21 antisense constructs.

Mutations in p53, a checkpoint protein that is best known as a regulator of genome stability based on its ability to halt cell cycle to allow DNA repair, characterize many tumors. In SCCHN and in the context of EGFR signaling, there are several specific ways in which p53 status has a critical role. A subset of SCCHN tumors is positive for HPV virus, which encodes the E6 oncogene, which promotes p53 degradation, reducing overall cellular levels of the protein. These tumors, arising predominantly in the oropharynx and tonsil, commonly lack disruptive

p53 mutations, while tumors from the oral cavity and larynx are HPV-negative and have disruptive 53 mutations [196]. Since Balaban *et al.* [197] documented a radioprotective effect of EGF during and after irradiation, the use of monoclonal EGFR antibodies to sensitize cancer cells to radiation has become one of the major treatment breakthroughs in the treatment of SCCHN, extending overall survival and cure rates. Among many factors that may play role in such differential radiation sensitization, the p53 inactivating mutations found in HPV-negative cancers may be important. Speculatively, in the absence of functional p53, EGFR-dependent proliferative effects may be less important, since disruptive mutations of p53 are strongly associated with amplification of, the EGFR-responsive target cyclin D1. Secondly, it is possible that induction of EGFR pathway components in response to radiation is compromised in cancers with disruptive p53 mutations. Radiation treatment of cells normally induces p53: one gene transcriptionally activated by p53 is HB-EGF, an EGFR activating ligand. This feedback pathway opposes and limits the extent of p53 growth suppression [198]. Clearly the situation is complex, as additional checkpoint proteins including p63 [199,200], p73 [201], and p14ARF [202], that have partially overlapping function with p53, are now also being implicated in the disease course of SCCHN, in some cases as regulatory targets of EGFR [191]. More study in this area is required.

2.3.3. STATs and SRC—The STAT (signal transducers and activation of transcription) proteins are important recipients of signals from the EGFR RTK family, and have attracted interest as therapeutic targets in SCCHN and other cancers [203,204]. STAT proteins are direct mediators of signaling from the cell surface to the nucleus, where they act as transcription factors. In cancer-relevant signaling, activation of STATs occurs based on joint action of an ErbB family protein (EGFR or ErbB2 are best studied) and the cytoplasmic tyrosine kinase c-Src. C-Src phosphorylates EGFR on Y⁸⁴⁵, creating a binding site for a STAT protein (STAT3 and STAT5b are most studied; Figure 2). The EGFR-bound STAT protein is directly phosphorylated by both c-Src and EGFR, activating its signaling function and promoting its translocation to the nucleus, and binding to promoters containing STAT-specific response elements.

Altered function of several STAT family members, including STAT1, STAT3, STAT5a and STAT5b, contributes to the development of human cancer [205], with STAT3 the first member of the family shown to have oncogenic potential [206]. SCCHNs commonly have constitutively active STAT3, and dominant-negative versions of STAT3 limit the growth rate of SCCHN cell lines [207]. Overexpression of STAT3 is also common in SCCHN, and is linked to high levels of cyclin D1 transcription [208]. STAT3 is a critical mediator of EGFR-induced proliferation [209]. Activation of STAT3 directly by EGFR results in activation of NFκB prosurvival pathway. STAT3 phosphorylation is increased in poorly differentiated SCCHN, and STAT3 levels correlate with nodal metastasis in SCCHN [210,211]. However, another study has found that STAT3 expression in primary SCCHN tumors did not predict outcomes [212], suggesting more study is required. Alternative signaling inputs may be involved in some cases: for example, Sriuranpong *et al.* have suggested that the constitutively active STAT3 in SCCHN may in some cases be independent of EGFR signaling [213], but instead be mediated by alternative upstream activators such as the cytokine IL-6 abundantly produced by SCCHN cell lines.

3. Targeting EGFR Resistance Networks in SCCHN

As summarized above, a network of proteins variously involved in direct interaction with EGFR, operating in closely coupled signaling pathways, or in complementary pathways influencing cell survival or damage checkpoints have been identified as relevant to SCCHN pathogenesis. A rational approach to improving therapies targeting EGFR or involving administration of cytotoxic agents or radiotherapy would be to consider the integrated

functioning of this signaling system as a whole network. Emerging therapeutic strategies combine EGFR-targeted and other targeted agents to disrupt this robust EGFR signaling network, to try to override compensating sources of therapeutic resistance. The following discussion touches on several approaches to combination therapy with EGFR antagonists and other targeted therapeutics.

3.1. EGFR inhibitors and STAT or SRC inhibitors

Although STATs have attracted interest as therapeutic targets, these efforts are still at an early stage (reviewed in [214]). Although no STAT inhibitor has been tested in the clinic for SCCHN, STAT3 has attracted interest as a therapeutic target in SCCHN, particularly with the idea the combination of EGFR-inhibition with a STAT inhibitor would be a productive therapeutic strategy [205]. Another interesting possibility in SCCHN is the interplay between PTEN and STAT3. de la Iglesia *et. al.* have demonstrated a PTEN-regulated STAT3 tumor suppressor pathway in glioblastoma [215]. As PTEN inactivation occurs frequently in SCCHN, this strategy has been proposed for SCCHN. Strategies simultaneously targeting EGFR and STAT3 have been effective in the preclinical models of SCCHN, both increasing apoptosis and reducing Bcl-xL expression [216]. One novel pharmacological strategy that inactivates STAT3 using decoy oligonucleotides mimicking its cognate binding sequence of the DNA [217] is now entering a phase I clinical trial (ClinicalTrials.gov, NCT00696176). Src inhibitors have been shown to be effective in NSCLC and SCCHN cell lines [218,219], and are now being evaluated in pre-clinical experiments and future clinical trials.

3.2. EGFR inhibitors and c-MET or IGF-IR inhibitors

Since EGFR and IGF-IR provide input into similar downstream signaling pathways (Figure 1), the compensatory activation of one receptor when the reciprocal receptor is blocked may be overcome by combining inhibitors EGFR with inhibitors of IGF-IR. Combined inhibition of IGF-IR and EGFR has proven successful in preclinical models [220,221]. Selection of the appropriate cancer for treatment with this drug combination is important, highlighted by the fact that one study found cooperativity of IGF-IR and EGFR inhibitors greater in tumor cell lines originating from cancers of epithelial, including SCCHN, as compared to mesenchymal origin [221]. This work, the IGF-IR kinase inhibitor PQIP synergized with erlotinib to cause apoptosis and growth inhibition, *in vitro*, and growth regression *in vivo*. Interestingly, in epithelial tumor cells in which this synergy was most pronounced, Akt was controlled cooperatively by EGFR and IGF-IR. Indeed, inhibition of EGFR or IGF-IR individually promoted the activation of the reciprocal receptor and EGFR inhibition shifted regulation of Akt from EGFR to IGF-IR.

Similarly, combined inhibition of c-MET and EGFR may be a useful strategy to counter stimulation of compensatory resistance pathways resulting from unilateral inhibition of one of these growth factor receptors. EGFR stimulation overcame the growth inhibitory effects of MET inhibition in MET amplified gastric cancer cells by restimulating MAPK and Akt signaling pathways, preventing loss of cyclin D1 expression and promoting cell cycle progression [222]. EGFR-mediated rescue of MET signaling inhibition was prevented when gefitinib was combined with PHA-665752, a selective MET inhibitor. Conversely, hyperactive MET signaling may rescue the growth inhibitory effects of EGFR inhibitors. As previously described, MET maintained EGFR phosphorylation in the presence of EGFR tyrosine kinase inhibitor by stimulating c-Src in SUM229 cells. Inhibiting MET in this EGFR-TKI resistant breast cancer cell line resulted in decreased EGFR tyrosine phosphorylation and cell growth in the presence of EGFR TKI [155]. The efficacy of combining c-MET and EGFR inhibitors in preclinical models of SCCHN remains to be evaluated.

3.3. EGFR inhibitors and mTOR inhibitors

Blockade of the mammalian target of rapamycin (mTOR) is under investigation in SCCHN. Targeting mTOR prevents activation of the essential resistance and growth signals emanating from PI3K and Akt and increases potency of the EGFR antagonists in resistant cancer cell lines. The signal transduction pathway involving PI3K/Akt and mTOR is associated with cellular nutrient regulation and proliferation and has been implicated in the regulation of angiogenesis via the increased activity of hypoxia inducible factor-1 alpha (HIF-1 α) and VEGF [223-225]. Jimeno *et al.* demonstrated synergistic antitumor effects of temsirolimus and erlotinib in resistant SCCHN cell line which were also paralleled by downregulation of MAPK, S6p70 phosphorylation and suppression of proliferation [226]. A study by Bianca *et al.* showed increased potency of gefitinib with another mTOR inhibitor, everolimus, in reducing proliferation and angiogenesis in combination with gefitinib [227]. This strategy was superior in inhibiting growth of GEO and GEO-GR (gefitinib resistant) colon cancer xenografts. This strategy has been tested in pancreatic cancer [228]. A phase I trial of the mTOR inhibitor, RAD001, in combination with cetuximab has been reported [229] and is also being investigated at Fox Chase Cancer Center.

3.4. EGFR inhibitors and inhibitors targeting other RTKs

Tumor-induced angiogenesis plays a role in the pathogenesis of head and neck cancer with up to 90% of head and neck cancers expressing VEGF or VEGFR. However, response rate of less than 4% are seen in SCCHN patients when small molecule anti-angiogenics such as sorafenib are used as monotherapies. Sunitinib and bevacizumab are currently being examined in ongoing trials [230]. Due to the density of interconnections of cell signaling pathways downstream of VEGFR and EGFR (Figure 1), targeted inhibition of both SCCHN-relevant RTK's was shown to be effective *in vitro* and *in vivo* in a preclinical mouse model [231-233]. Encouragingly, a response rate of 14.6% was seen when erlotinib was combined with bevacizumab, a monoclonal antibody against VEGF-A [230].

Additional RTKs have been proposed to play a role in SCCHN. Notably, the other EGFR family RTKs, ErbB2, ErbB3, and ErbB4, have been examined in SCCHN. At the pre-clinical investigation level, ErbB2 is the most well studied in SCCHN. Overexpression of ErbB2 has also been linked with resistance to gefitinib, while combination of the ErbB2-targeting agent pertuzumab with gefitinib increased growth inhibition in resistant SCCHN cell lines [143]. Potent therapeutics to target ErbB2 are available, and targeted therapeutics against ErbB2 such as trastuzumab are standard treatments for breast cancers with ErbB2 over-expression and amplification [234]. A gefitinib-trastuzumab combination was effective in increasing growth inhibition of ErbB2-positive SCCHN cell lines [235]. There is as yet little documented evidence of successful clinical use of ErbB2-targeting agents in SCCHN. However, one intriguing recent study suggesting a combined signature of gains in EGFR, ErbB2, and loss of CDKN2A effectively predicts resistance to the EGFR- and ErbB2-targeting agent lapatinib in 8 of 10 SCCHN cell lines [236]. Although ErbB3 and ErbB4 have been studied in head and neck cancer cell lines, the use of combination therapies with EGFR antagonists remains investigational.

4. Conclusion

The modest results of EGFR antagonists in SCCHN despite the dependence of head and neck cancers on this EGFR signaling prompted investigators to characterize the variety of EGFR resistance pathways discussed in this review (Figure 4). As illustrated above, an increasingly detailed knowledge of EGFR signaling relationships has begun to allow the development of rationally designed combination therapies that have begun to improve effective use of EGFR inhibitors in the clinic. A more comprehensive understanding of EGFR resistance pathways will provide the basis for novel targeted therapeutics. Because of the expense and length of

time required to establish new trials, a number of promising combinations predicted based on preclinical work have not yet been assessed in humans, raising the potential for significant clinical gains as these combinations move through the Phase I/Phase II process. Although many of these combinations are more likely to yield incremental rather than transformative therapeutic gains, nevertheless, the wealth of potential combinations becoming available also suggests that by steps, ultimately significant gains will emerge.

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Figure 1. Resistance mechanisms originating from parallel growth factor receptors

Growth factor receptors such as vascular endothelial growth factor receptor (VEGFR), insulin-like growth factor I receptor (IGF-IR), mesenchymal-epithelial transition factor (c-MET) provide compensatory activation of cell survival and proliferation pathways when EGFR is inhibited. Acting through a common adaptor molecule, Grb2, IGFIR, c-MET and EGFR drive proliferation and cellular survival signals by activating the Ras>Raf>MEK>Erk axis. VEGFR also activates this signaling cascade via the phospholipase C-γ (PLC-γ) mediated activation of protein kinase C (PKC) which in turn activates Raf. The phosphatidylinositol 3-kinase-protein kinase B (PI3K-Akt) signaling axis is activated by either direct association of PI3K to the cytoplasmic domain of tyrosine kinase receptors or via adaptor molecules such as insulin receptor substrate 1 (IRS1) or Grb2-associated binding protein 1 (Gab1). Signal Transducers and Activator of Transcription (STAT) are phosphorylated by EGFR, EGFR-interactor Src, and adjacent growth factor receptors (c-MET). The phosphorylated forms of STAT translocate to the nucleus and activate the transcription of genes involved in cell cycle progression, angiogenesis and apoptotic resistance. Other members of the ErbB family can compensate for inhibition of EGFR signaling by dimerizing amongst themselves to activate an overlapping set of downstream effectors. mTor denotes mammalian target of rapamycin; Sos, son of sevenless; Grb2, growth factor receptor-bound protein 2; MEK, MAPK kinase; Erk, extracellular signal-regulated kinase.

Figure 2. Resistance mechanisms originating from EGFR downstream effectors

Cell signaling effectors downstream of EGFR may mitigate the efficacy of EGFR inhibitors by maintaining signaling activity to drive cell cycle progression and inhibit apoptosis. Increased signaling of the PI3K-Akt pathway, STAT proteins and cell cycle progression mediators such as Cyclin D1 have been associated with resistance to EGFR inhibitors in SCCHN. See text for details.

Figure 3. Regulatory mechanisms operating at the level of the EGFR receptor and its ligands

I. Overexpression. Overexpression of EGFR in HNSCC has been linked to resistance to EGFR inhibitors. Mechanisms resulting in overexpression include increased *EGFR* copy number and mutations at the level of *EGFR* gene or promoter. **II. Kinase domain mutations.** Kinase domain mutations include in-frame deletions and amino acid substitutions centering around the ATP binding cleft of EGFR. These mutations lead to increased ligand-dependent activation of EGFR and increased sensitivity to EGFR inhibition by allowing easier access for both ATP substrate and competitive inhibitor. The prevalence of kinase domain mutations in head and neck cancer is low. **III. EGFR vIII.** EGFRvIII is a constitutively active form of the receptor, and has been associated with resistance to EGFR inhibitors in many SCCHNs. **IV. Glycosylation.** Glycosylation of EGFR contributes to ligand-induced receptor activation. In certain contexts, glycosylation status of EGFR may modify response to EGFR-targeted antibody and small molecule inhibitors. **V. Ligand availability.** The ADAM family of sheddases catalyzes the proteolytic reaction required for the releases the transmembrane precursors of EGFR ligands. Activation of ADAM-17 results in release of amphiregulin and is associated with activation of EGFR in HNSCC. In addition, amphiregulin expression predicts the sensitivity of SCCHN to inhibition by gefitinib and cetuximab. **VI. Nuclear EGFR.** Nuclear EGFR was shown to activate the transcription of the cell cycle progression mediator, cyclin D1. The mechanism of nuclear translocation and its importance as a sensitivity mediator to clinical EGFR inhibitors remains an area of active investigation.

Figure 4.

a. EGFR signaling drives cell survival and proliferation signals. EGFR transmits cell survival and proliferation signals through multiple downstream signaling pathways. Signals from EGFR are amplified due to both the density of interconnections in downstream signaling pathways and the parallel input provided from other membrane-bound growth factor receptor tyrosine kinases (RTKs).

b. Signaling mediators parallel or downstream to EGFR compensate for EGFR inhibition and limit the clinical efficacy of EGFR inhibitors. Inhibition of EGFR with targeted therapeutic antibodies or small molecule inhibitors has only limited clinical success. Resistance to EGFR inhibition develops due to the maintenance of cell survival and proliferation signals by activation of signaling effectors such as insulin-like growth factor I receptor (IGF-IR) which are parallel to EGFR or signaling effectors such as phosphoinositide-3-kinase (PI3K) which are downstream of EGFR.

c. Rational drug combination strategies are required to overcome EGFR resistance. Resistance to EGFR inhibitors in head and neck cancer may be overcome by treating patients with a combination of EGFR inhibitors and inhibitors of biological targets such as RTKs parallel to EGFR or protein kinases downstream of EGFR. Combined inhibition of EGFR and

an EGFR-resistance mediator such as IGF-IR or PI3K will synergistically decrease cell survival and proliferation signals. In cancer cells which are dependent on EGFR signaling, such a combination drug treatment can cause cell cycle blockade and initiate apoptosis by increasing pro-apoptotic signals and decreasing anti-apoptotic signals. Thorough understanding of molecular mechanisms of EGFR resistance in an individual tumor is required to choose the correct combinational target and optimize the clinical efficacy of EGFR inhibitors.