Chapter 2
Targeting the ErbB Family in Head and Neck Cancer

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Abstract Members of the ErbB receptor tyrosine kinase family (EGFR, HER2, HER3, and HER4), which regulate cell differentiation, proliferation, and survival, are commonly overexpressed and hyperactivated in squamous cell carcinoma of the head and neck (SCCHN). This abnormal expression and activity triggers multiple effector cascades that promote cancer growth, involving signaling through Ras-Raf-ERK1/2, PI3K/AKT/mTOR, JAK1/STAT3, PLC/PKC, and others. Targeting of EGFR remains one of the most common therapies for patients with SCCHN, with newer therapies also targeting additional ErbB family members and ErbB effectors, and exploring combinatorial approaches. In this chapter, we will describe the biology of ErbB family receptors in normal cells and in SCCHN, current and novel therapeutic approaches, and mechanisms underlying resistance to anti-EGFR therapy.

Keywords Head and neck cancer · ErbB family · EGFR. · EGFR-targeted therapy · Anti-EGFR therapy resistance

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2.1 Introduction

In the past decade, protein-targeted inhibitors have become valuable tools in the treatment of squamous cell carcinoma of the head and neck (SCCHN). The epidermal growth factor receptor (EGFR), also known as avian erythroblastic leukemia viral (v-erb-b) oncogene homolog 1 (ErbB1) or human epidermal receptor (HER1), was one of the first targetable proteins identified as relevant to SCCHN [1, 2]. Subsequent broadened research and development efforts have expanded the armament to encompass agents that also inhibit EGFR family members ErbB2/HER2 (sometimes designated neu), ErbB3/HER3, and ErbB4, as well as key downstream effectors including RAF and phosphoinositol-3-kinase (PI3K).

The ErbB family was first identified as cancer-relevant in the 1980s when an aberrant form of the human epidermal growth factor (EGF) receptor was found to be encoded by the avian erythroblastosis tumor virus [3]. The four members of the ErbB family are structurally related with each containing a large extracellular N-terminal region, a single hydrophobic transmembrane-spanning domain, an intracellular juxtamembrane region, a tyrosine kinase domain, and C-terminal region [4–6]. ErbB3 differs from the other family members in having a kinase domain that was long thought to be a pseudokinase although it has now been shown to have weak autophosphorylation capacity [7] and, through heterodimeric interactions, to serve as an activator of the EGFR kinase domain [8]. Importantly, members of the ErbB family function as homodimers and heterodimers [9, 10]. In normally growing cells, dimer formation and signaling typically involve activating interactions between the extracellular N-terminal domain and small ligands (discussed in Sect. 2.2). Interactions between dimer subunits induce essential phosphorylations within the ErbB C-terminal regions that provide binding sites for partners that interact with effector proteins to initiate downstream signaling cascades and initiate feedback signaling that ultimately restricts ErbB signaling activity. Key effectors that are activated as a result of these phosphorylations include PI3K, PLCγ, GRB2, c-SRC, and JAK. Cyclic, transient activation of ErbB family signaling in normal cells is regulated by a number of factors, including ligand availability, cytoplasmic phosphatases, and the endocytic/degradation machinery (Sect. 2.2).

In human SCCHN, activation of EGFR and its family members occurs by several distinct mechanisms (discussed at length in Sect. 2.3). Elevated expression of EGFR was originally described as characterizing 80–90% of SCCHN [11, 12], and several studies have indicated that overexpression of EGFR correlates with resistance to therapy and reduction of overall survival (OS) [13–15]. However, a meta-analysis evaluating EGFR prognostic value has demonstrated that EGFR overexpression correlates with OS, but not disease-free survival (DFS) [16], and additional recent studies suggest a more complicated relationship between overexpression and survival (Sect. 2.3.1). Although EGFR is by far the most commonly overexpressed ErbB family member in SCCHN, the three other members are also overexpressed in a significant number of cases (ErbB2/HER2, 3–29%; ErbB3/HER3, 21%; and ErbB4/HER4, 26%; [17]). Moreover, ligands contributing to the activation of ErbB proteins are overexpressed in some SCCHN tumors (Sect. 2.3) [18]. In addition, mutational activation of some critical effectors, such as PI3K, defines a subset of

SCCHN [19]. Finally, the past decade has been marked by a growing appreciation of differences in biology and prognosis associated with the presence or absence of human papillomavirus (HPV) as an oncogenic driver in SCCHN [20, 21], and some evidence suggests that HPV status may influence expression and activity of the ErbB proteins [22, 23].

Activation of the ErbB family of transmembrane receptor tyrosine kinases (RTKs) and their downstream effectors is typically associated with rapid cellular growth, as well as activation of the DNA repair machinery induced by DNA-damaging therapies commonly used in treatment of SCCHN, contributing to resistance to cytotoxic therapies such as cisplatin or radiation [14]. Based on abundant evidence, therapeutics targeting the ErbB family and its effectors have appeared to be particularly appropriate for the treatment of SCCHN. Two complementary therapeutic strategies have been developed to target EGFR and its family members. A first strategy involves targeting the extracellular domain of the receptor with monoclonal antibodies, such as cetuximab, panitumumab, zalutumumab, and others that interfere with the processes of dimerization and activation of the intracellular kinase domains [24, 25] (Sect. 2.4.1). A second strategy targets the intracellular domain of the receptor with low-molecular-weight tyrosine kinase inhibitors (TKIs; e.g., gefitinib and erlotinib; see Sect. 2.4.2) [26]. More recently, therapeutic strategies have expanded to include the use of drugs or drug combinations that target multiple ErbB family members or that combine ErbB-targeting drugs with those targeting critical downstream effectors, such as PI3K or MEK1 (Sect. 2.3.3.2) [27]. The nature of EGFR/ErbB signaling and therapeutic strategies to manage tumors with EGFR/ErbB involvement are addressed in detail in the remainder of this chapter.

2.2 Regulation of EGFR and the ErbB Family in Normal Cells

2.2.1 Ligand Binding and Dimerization: Activation of ErbB Proteins in Normal Cells

The extracellular regions of ErbB family members contain two homologous ligand-binding domains (domains I and III) and two cysteine-rich domains (domains II and IV). The ligands required for dimerization and activation of EGFR, ErbB3, and ErbB4 can be separated into five groups: (1) EGFR-specific ligands such as EGF, amphiregulin (AR), epigen (EPN), and transforming growth factor alpha (TGFα); (2) the ErbB3-specific ligands neuregulin1α (NRG1α), NRG2α, and NRG6; (3) NRG3, NRG4, and NRG5 that specifically bind ErbB4; (4) the bispecific ligands betacellulin (BTC), epiregulin (EPR), and heparin-binding EGF-like growth factor (HBEGF), which bind EGFR and ErbB4, and NRG1β which binds ErbB3 and ErbB4; and (5) NRG2β, which is a pan-ErbB ligand and binds to EGFR, ErbB3, and ErbB4 [28] (Fig. 2.4). Uniquely, ErbB2 does not depend on ligands for dimerization or activation. Instead, domains I and III interact directly in a configuration that renders the ligand-binding site inaccessible [29–31]. To date, no high-affinity soluble

ligand has been identified for ErbB2 [29, 32]. It is possible that assignment of ligand specificity is not exact; for example, a recent study has demonstrated that stimulation of ErbB4 with NRG1 activates the transcriptional activator YAP, promoting YAP-dependent cell migration [33].

ErbB proteins can homodimerize or heterodimerize [34]. EGFR-EGFR and ErbB4-ErbB4 homodimers and EGFR-ErbB2, EGFR-ErbB3, ErbB2-ErbB3, and ErbB2-ErbB4 heterodimers are abundant in SCCHN tumors and cell lines [17, 35, 36]. There is also evidence that activation of the catalytic domain of EGFR through homo- or heterodimerization occurs due to its increased accumulation at the plasma membrane and can be enhanced by a common mutation in a leucine (*L834R*), which suppresses local disorder [37] and is associated with drug resistance in some tumor types [38]. Some similarities of the EGFR kinase domain with Src and cyclin-dependent kinase (CDK) domains have been observed that support an alternative mechanism for dimerization, in which one EGFR kinase domain interacts asymmetrically with the second domain in a dimer pair, as a cyclin activates a CDK [9].

The configuration changes associated with dimerization lead to transient kinase activation in normal cells. These become constitutive in cancers, in the setting of kinase overexpression. The actual activation process involves an asymmetric interaction between intracellular kinase domains that results in auto- or transphosphorylation of ErbB family members [8, 39, 40]. As ErbB2 is not ligand-responsive, phosphorylation of this kinase can be activated through homodimerization [41, 42] or heterodimerization, frequently with ErbB3 [7, 8]. EGFR and ErbB4 can function independently of other ErbB receptors and autophosphorylate C-terminal tails after binding to activating ligand. These phosphorylations provide binding sites for proteins that transduce activating signals downstream (e.g., STAT5b, GRB2, SHC, GAB1/PI3K(p85), PLCγ), which induce signaling relevant to proliferation, apoptosis resistance, and DNA synthesis [43].

2.2.2 *ErbB Trafficking and Other Mechanisms to Limit EGFR Function in Normal Cells*

As with most RTKs, duration of ErbB activation is limited by countervailing regulatory processes. Some of the phosphorylations on the C-terminal domains of the ErbB proteins provide binding sites that allow feedback signaling that downregulates the activated ErbB protein through dephosphorylation, ubiquitination, and/or internalization (e.g., SHP1, CBL, CRK). More than one pathway for internalization has been described. In the most studied pathway, binding of the E3 ubiquitin ligase Cbl to phosphorylated Y-1045 of activated EGFR at the plasma membrane triggers clathrin-mediated endocytosis [44]. Multiple additional activation-associated phosphorylations conferred by calmodulin kinase II and p38 enhance the interaction of Cbl with activated EGFR [45, 46].

Subsequently, during EGF-mediated endocytosis, EGFR is either recycled to the plasma membrane or alternatively processed through the late endosome and multi-

vesicular body for proteolytic degradation in the lysosome [47]. An alternative non-clathrin-based endocytotic process has also been described: in this case, the majority of EGFR is targeted for lysosomal destruction [48, 49]. Additional interactions involving the molecular motor dynamin 2 (DYN2) and a scaffolding protein, CIN85, support targeting of EGFR to the lysosome rather than for recycling [50]. As discussed below, reduced phosphorylation of EGFR that limits interaction with Cbl and other internalization proteins often accompanies therapeutic resistance to EGFR inhibitors (EGFRIs). EGFR may also undergo ligand-independent internalization through p38MAPK- and clathrin-mediated, or Src- and caveolin-mediated mechanisms, upon conditions of cell stress [51]. Dutta et al. have recently reported that neuropilin-2 (NRP2) plays an important role as an endocytotic regulator for EGFR, with depletion of NRP2 disrupting normal regulation of endocytic transport of EGFR from the cell surface and leading to the accumulation of active EGFR in endocytic vesicles and abnormal ERK activation [52].

Extending understanding of traffic controls, compartmentalization of the EGFR partner ErbB2 is controlled by a member of the Anks1a adaptor protein family in the endoplasmic reticulum (ER). Within the ER, an ErbB2 complex with another RTK, ephrin A2 (EphA2), allows binding to Anks1a, which in turn regulates EphA2/ErbB2 complex exit. This process is positively associated with tumorigenesis [53]. Once activated, ErbB2 remains at the cell surface, potentially due to an interaction with HSP90 or the plasma membrane calcium ATPase2 (PMCA2) [54, 55]. In breast cancer, inhibition of PMCA2 disrupts binding between ErbB2 and HSP90, leading to ErbB2 internalization and degradation. In MMTV-Neu mice, PMCA2 knockout effectively inhibits tumor formation, suggesting that interaction of ErbB2 and PMCA2 is a potential therapeutic target for this and other cancer types [54] (Fig. 2.1).

2.3 Causes and Consequences of Altered EGFR/ErbB Function in SCCHN

2.3.1 Overexpression of EGFR and Its Ligands

The degree to which EGFR is overexpressed in SCCHN has been reported differently by different groups, reflecting varying approaches used to measure DNA amplification, mRNA overexpression, and protein overexpression, the use of different cutoff values, and (potentially) differences in EGFR expression based on SCCHN sub-site (e.g., oral cavity versus laryngeal). EGFR overexpression in SCCHN is often caused by an increase in the number of gene copies [56] but can also occur at the mRNA or protein level. The original studies reporting overexpression of EGFR in 80–90% of SCCHN [11, 12] were based on analysis of mRNA expression in a limited set of 24 tumor specimens and 10 SCCHN cell lines versus histologically normal mucosa specimens. Follow-up work by Grandis and Tweard found that median EGFR mean optical density (based on IHC analysis using preparations of the EGFR-overexpressing A431 cell line as a positive control) was 54%

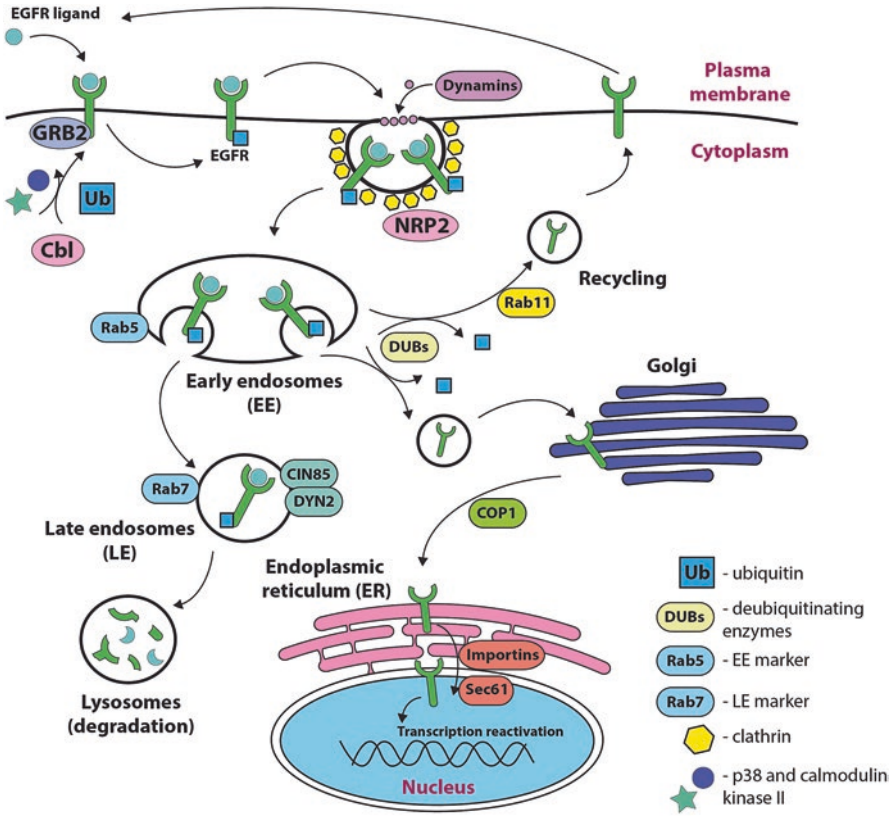


Fig. 2.1 Regulation of internalization and degradation of ErbB proteins. Here we described clathrin-mediated endocytosis of EGFR with further transportation to late endosomes and degradation, recycling, or migration to the nucleus

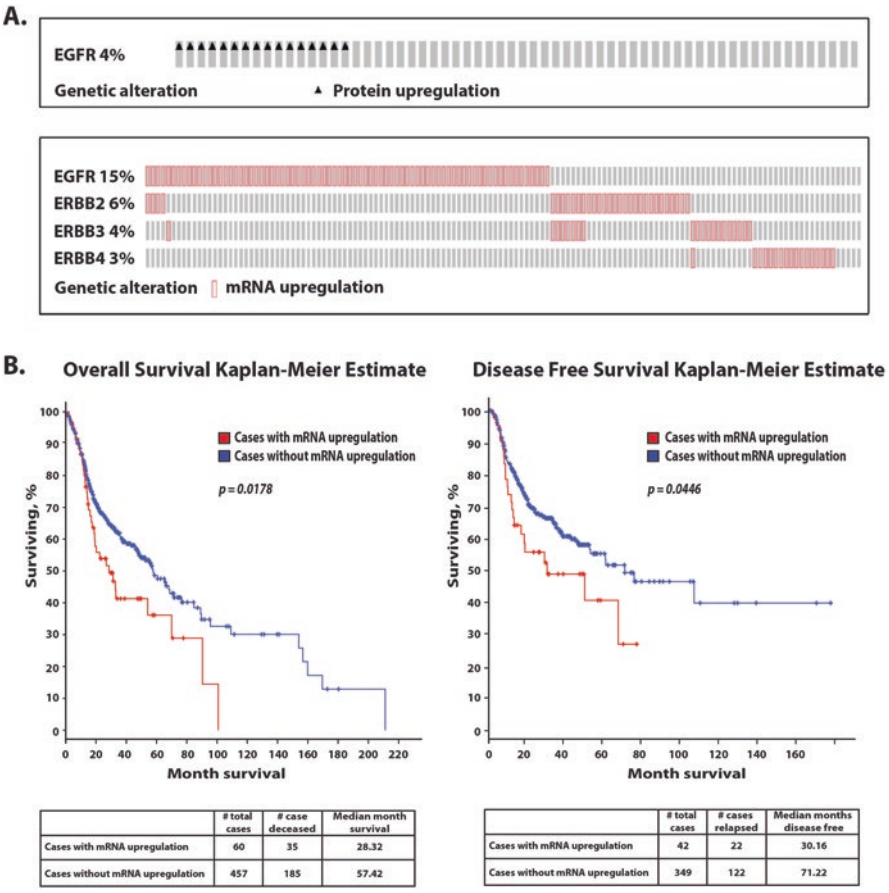
in a group of 91 patients with SCCHN [57]. In another study, EGFR protein levels in 140 primary laryngeal squamous cell carcinomas were determined using a radioligand receptor assay. The authors established that a cutoff value of 20 fmol mg^{-1} is an effective prognostic marker, and based on this classification, 28 of 140 patients (20%) had elevated levels of EGFR and lower 5-year survival (25%) in comparison with patients with EGFR levels <20 fmol mg^{-1} (81% 5-year survival) [58]. Poor prognosis was also associated with an increased copy number of *EGFR*. *EGFR* gene copy numbers were analyzed in 134 SCCHN tumors using quantitative PCR, with this study finding aberrant *EGFR* copy numbers in 24% of tumors, with 17% of tumors having increased copy numbers [59].

Ongkeko et al. used IHC analysis to show that EGFR was highly expressed (38%–43%) in 21 pharyngeal, 16 laryngeal, and 1 floor of mouth carcinoma compared with benign samples [60], based on qualitative rankings from 3 independent pathologists. Using immunohistochemistry (IHC), Bei et al. found EGFR to be overexpressed in 47% of cases in a group of 38 SCCHN tumor samples in comparison with 24 adjacent normal mucosa specimens [61]. Bernardes et al. analyzed

EGFR in a subset of 52 patients with oral squamous cell carcinoma (OSCC) using 3 different methods: IHC, fluorescent in situ hybridization (FISH), and chromogenic in situ hybridization (CISH). This study showed that EGFR overexpression rates were 53.8% (28/52) by IHC, 5.8% (3/52) by CISH, and 15.4% (8/52) by FISH [62]. Pectasides and colleagues found that increased gene copy number did not directly correlate with protein expression of EGFR, and elevated protein levels of EGFR determined by IHC better correlated with the poor clinical outcome than did *EGFR* copy number determined by FISH [63]. Ang et al. demonstrated that EGFR expression varied widely in a group of 155 patients (based on automatic IHC analysis) and that higher EGFR expression in SCCHN samples correlated with reduced OS and DFS (based on the mean optical density data) [15].

The increasing availability of systematic genomic profiling provides additional data points [64], but does not resolve the issue of how to best define EGFR overexpression values. Among the 357 SCCHN specimens analyzed by The Cancer Genome Atlas (TCGA) Consortium for which genomic data are available, based on default TCGA analysis settings, *EGFR* amplification occurs in 4% of tumors. Among 520 SCCHN specimens with mRNA expression data collected by the TCGA, upregulation at the mRNA level is seen in 15% of cases. Based on these expression data, upregulation results in shorter overall and disease-free survival (z-score >2.5, OS, 28.32 months, versus 57.42 months; DFS, 30.16 months, versus 71.22 months) (Fig. 2.2) [65, 66]. In contrast, a recent study reporting genetic and molecular profiling by Caris Life Sciences of 123 and 236 patients with advanced SCCHN demonstrated that EGFR was overexpressed in 90% of cases by IHC but only 21% by ISH, respectively [67].

Increased EGFR expression is not only found in SCCHN tumor samples but has also been observed in "healthy" mucosa samples of patients with SCCHN [68] and likely reflects a premalignant event in the tissue adjacent to an incipient SCCHN [11, 12]. Hence, elevated EGFR expression is a potential biomarker for early stages of malignant transformation in addition to being a therapeutic target [69]. Similarly, a study of 155 patients found that EGFR expression did not correlate with disease stage at presentation, or other known clinical prognostic variables, in stage II–IV carcinomas of the oral cavity, oropharynx and supraglottic larynx, tongue base, and hypopharynx, although EGFR expression was an independent prognostic indicator of 5-year OS (40% for EGFR negative and 20% for EGFR positive; $p = 0.0006$) as well as disease-free survival (DFS) (25% for EGFR negative and 10% for EGFR positive; $p = 0.0016$) [15]. These findings agreed with an earlier study, based on 140 primary laryngeal squamous cell carcinomas, where the 5-year survival rate was 81% for patients with tumor cells defined as EGFR negative based on biochemical assessment of EGF-binding capacity of membrane fractions prepared from tumors, compared to only 25% for patients with EGFR-positive tumors [58]. This study also reported 5-year relapse-free survival (RFS) of 77% for patients with EGFR-negative tumors, compared to 24% for patients with EGFR-positive tumors [58]. Chang et al. have shown that high EGFR expression also correlates with treatment failure in early glottic cancer treated with radiation alone and that EGFR expression is higher in the tumors of patients with recurrent disease than in controls [70]. In one study, using immunohistochemical (IHC) analysis, EGFR distribution within the tumor



	# total cases	# case deceased	Median month survival
Cases with mRNA upregulation	60	35	28.32
Cases without mRNA upregulation	457	185	57.42

	# total cases	# cases relapsed	Median months disease free
Cases with mRNA upregulation	42	22	30.16
Cases without mRNA upregulation	349	122	71.22

Fig. 2.2 TCGA data for EGFR in SCCHN cancer specimens. (**a**) A representative map of data illustrating TCGA data for EGFR upregulation at the protein level (*top* panel) and mRNA levels for EGFR and other ErbB family members in SCCHN specimens.(**b**) Kaplan-Meier survival curves comparing the SCCHN patients with (*red*, z-score > 2.5) and without (*blue*, z-score < 2.5) EGFR mRNA level upregulation. Overall survival (OS), p-value = 0.0178; progression-free survival (PFS), p-value = 0.0446

tissue was related to patient survival, with heterogeneous distribution of EGFR in tumors significantly associated with poorer OS and DFS, in comparison with homogeneous distribution [71].

Additional members of the ErbB receptor family have been detected in SCCHN at increased expression levels [72, 73], although conflicting reports regarding ErbB3 and ErbB4 expression levels have been published [61, 72, 74]. In the TCGA, protein overexpression was observed for ERBB2 in 2.2% of tumors and for ERBB3 in 5% of tumors: this represents too few cases to perform meaningful analyses and determine potential correlation with survival.

Changes in EGFR and ErbB family internalization and degradation mechanisms have also been associated with SCCHN. Changes in these mechanisms associated

with cancer lead to membrane accumulation of ErbB, further contributing to the abnormal activation of EGFR/ErbB signaling, which potentially promotes tumor formation and progression [75]. In this regard, a recent study revealed the relationship between the lysosomal enzyme cathepsin S (CTSS) and EGFR signaling regulation, with increased expression of CTSS detected in a number of types of cancer. Inhibition of CTSS limited EGFR degradation and caused EGFR accumulation in the late endosomal and in the perinuclear region, leading to formation of spatial compartments with extended EGFR, STAT3, and AKT signaling. Combined treatment with the EGFR inhibitor gefitinib and the CTSS inhibitor 6r also significantly increased cellular apoptosis [76]. Downregulation of c-CBL, which mediates internalization and degradation of EGFR, has been identified in a significant subset of SCCHN tumors [77]. Reciprocally, upregulation of the HECT-class ubiquitin ligase SMURF2, which ubiquitinates EGFR in a manner that protects it from c-CBL-dependent degradation, has also been suggested to be important in SCCHN [78].

Upstream of EGFR, overexpressions of ligands such as TGFα have been linked to a poor prognosis [68, 79] and have been associated with malignant tumor development at a number of sites in transgenic mice [80–82]. Additionally, expression of TGFα [17], AR [83, 84], and HB-EGF [85] has been shown to enhance oncogene-induced carcinogenesis and affect the response of tumor cells to EGFR inhibition [86–89], with some evidence suggesting other ligands are likely to also be of importance [90]. Elevated expression of mRNAs for EGFR ligands including AREG (amphiregulin), EGF, HB-EGF, and betacellulin (BTC) was associated with reduced patient survival [91]. Some proteins, such as the CBL-interacting protein of 85 kDa (CIN85), which regulates EGFR internalization, have been shown to be overexpressed in some advanced SCCHN and to increase TGFα-dependent signaling in SCCHN tumors [92].

As Brand et al. have reviewed in detail [93], epithelial cancers such as SCCHN are, surprisingly, characterized by a high frequency of nuclear EGFR localization. Mechanistically, to enter the nucleus, EGFR is passaged from clathrin-coated pits to the Golgi and subsequently via retrograde transport in COPI vesicles to the endoplasmic reticulum (ER) [94], after which the Sec61 translocon moves EGFR from the inner nuclear membrane to the nucleus [95, 96] (Fig. 2.1). Nuclear EGFR acts as a transcription coactivator for many genes associated with cell proliferation, including BCRP, Aurora-A, cyclin D, Myc, c-Myb, Cox-2, and iNOS, and also binds and supports activity of PCNA and DNAPK to enhance DNA synthesis and repair [97]. Increased expression of nuclear EGFR has been associated with a higher incidence of local recurrence and inferior DFS in oropharyngeal squamous cell carcinoma [98, 99]. Nuclear EGFR expression levels retained their prognostic significance in multivariate analysis adjusting for well-characterized prognostic variables [99]. Saloura et al. have reported that posttranslational methylation of the tyrosine kinase domain of EGFR by methyltransferase WHSC1L1 increased activation of the ERK pathway in the absence of EGF stimulation. Interestingly, this methylation appeared to be important for nuclear EGFR, promoting its interaction with PCNA (proliferating cell nuclear antigen) in SCCHN cells and enhancing DNA synthesis and cell cycle progression [100]. At present, it is not clear whether this localization is unique to cancer cells or instead represents an extreme case of a signaling process that also exists in normal cells: in general, this phenomenon requires further study.

2.3.2 Alternative Forms of EGFR and Its Effectors Affecting Signaling Activity in SCCHN

It has been suggested that expression of truncated and activated EGFR is associated with advanced tumor and nodal stage [101]. In studies of SCCHN tumors, Hama et al. detected only 5 different *EGFR* mutations in 6 out of 82 patients [102]. Additional ErbB family members were not identified as commonly mutated in either of these studies. A meta-analysis of multiple studies, including 4122 patients with SCCHN, suggested a 2.8% frequency of mutations affecting the tyrosine kinase domain [102, 118, 119]. Another two studies identified *EGFR* mutations in only 3 of 127 patients (2.4%) and 17 of 110 (16%), respectively [103, 104]. A fourth study found an in-frame deletion mutation in exon 19 of *EGFR* (E746_A750del) in 3 of 41 larynx, tongue, and tonsil tumor samples [105].

One *EGFR* mutation of note reported in SCCHN is EGFR variant III (EGFRvIII), which results in a truncation of the ligand-binding domain that results in ligand-independent, constitutive signaling, greatly potentiating tumorigenicity. EGFRvIII is the most common form of mutant *EGFR* and has been described in several types of cancer [106–111], including SCCHN [102, 112, 113]. However, the reported frequency of EGFRvIII in head and neck cancer is highly inconsistent. The presence of EGFRvIII in SCCHN ranged from none [102] to 15% [114] to 42% [113] and may vary by specific SCCHN subsite [115]. Sok et al. reported that EGFRvIII-transfected SCCHN cells showed increased proliferation in vitro and increased tumor volumes in vivo compared with vector-transfected cells. Furthermore, EGFRvIII-transfected SCCHN cells showed decreased apoptosis in response to cisplatin and decreased growth inhibition following treatment with cetuximab compared with vector-transfected control cells. However, it was not established if the transfected cells expressed EGFRvIII at levels similar to those observed in actual patient samples, given conflicting results in different studies [113, 116]. The significance of this variant remains unclear.

Stransky et al. performed whole exome sequencing on tumor samples from 92 patients with SCCHN and validated known relevant mutations in *TP53*, *CDKN2A*, *PTEN*, *PIK3CA*, and *HRAS* [117]. Agrawal et al. used the same methods to study 32 primary tumors, and 6 of the genes that were mutated in multiple tumors were reassessed in up to 88 additional SCCHN samples. This study identified mutations in *FBXW7* and *NOTCH1* in addition to previously identified genes [118].

Li et al. compared the genomic data of 39 SCCHN cell lines with genomic findings from 106 SCCHN tumors. Their results indicated that eight genes (*PIK3CA, EGFR, CCND2, KDM5A, ERBB2, PMS1, FGFR1*, and *WHSCIL1*) are amplified and five genes (*CDKN2A, SMAD4, NOTCH2, NRAS*, and *TRIM33*) are deleted in both SCCHN cell lines and tumors. Among the mutated genes relevant to the ErbB pathway, activating mutations of the catalytic subunit of *PI3K (PI3KCA)* were shared both in cell lines and in tumors – a result confirmed by a number of other studies [117–120] – and, importantly, based on the pharmacologic profiling results of eight anticancer agents, these mutations influence drug resistance [121].

2.3.3 Consequences of EGF/ErbB Activation

Dimerization of the ErbB RTKs can result in the constitutive activation of a number of intracellular signaling pathways, each of which contributes to the oncogenic activity of this kinase family in SCCHN. Some of the better-studied and physiologically significant effector pathways are represented in Fig. 2.3 and discussed below.

2.3.3.1 Ras/Raf/MAPK

Increased activity of the Ras/Raf/MAPK pathway initiated by EGFR signaling is strongly linked to tumorigenesis in SCCHN [94]. Following EGFR autophosphorylation, mainly on residues Y1068 and Y1086, the growth factor receptor/bound protein 2 (GRB2) adaptor protein is either directly recruited through binding of its Src homology 2 (SH2) domain to the phosphotyrosine residues of the activated receptor or, alternatively, GRB2 is indirectly recruited to active EGFR by interaction with the Src homolog and collagen homolog (SHC) adaptor protein, which directly binds tyrosine-phosphorylated sites on EGFR, itself is tyrosine phosphorylated, and then binds GRB2 [95]. EGFR-bound GRB2 subsequently recruits and activates guanine nucleotide exchange factor Son of Sevenless (SOS). Activated SOS increases the pool of active, GTP-bound Ras, inducing a kinase cascade involving c-Raf, MEK1/2, and ERK1/2 (Fig. 2.3). Phosphorylated ERK1/2 translocates into the nucleus and activates transcription factors that induce transcription of many genes promoting cell growth and survival; a residual pool of active cytoplasmic ERK1/2 also phosphorylates cytoskeletal proteins such as actin, which promotes cell motility, and regulators of cell division and cytokinesis, vesicle and organelle movement, and mitochondrial targets such as Bcl2 that render cells resistant to apoptosis (Fig. 2.3) [95, 96].

2.3.3.2 PI3K/Akt/mTOR

Dimerization of EGFR or ErbB2 with ErbB3 is strongly associated with PI3K activation, because of the high prevalence of PI3K-activating docking sites on ErbB3 [97]. PI3K proteins are composed of a catalytic p110 and a regulatory p85 subunit. The p110 subunits catalyze the phosphorylation of phosphatidylinositol 4,5-diphosphate (PIP2) to the second-messenger phosphatidylinositol 3,4,5-triphosphate (PIP3), which in turn phosphorylates and activates the protein serine/threonine kinase AKT (also known as protein kinase B), inducing protein synthesis and cell growth through activation of the mTOR effector pathway and limiting the apoptotic machinery [98]. AKT activation may also be induced by the binding of serine protease inhibitor Kazal-type 6 (SPINK6) to the EGFR

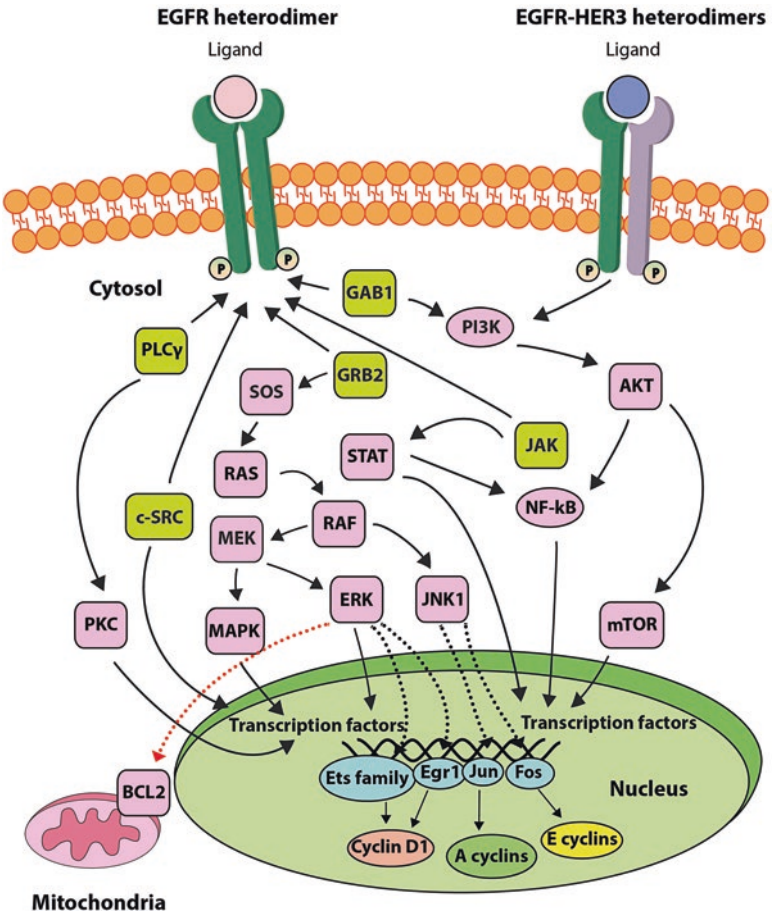


Fig. 2.3 Signaling pathways downstream of EGFR and other ErbB proteins that have been linked to tumorigenesis of SCCHN and/or resistance to ErbB-targeting inhibitors. *Green* boxes indicate targets which bind directly to the EGFR phosphorylation sites. See text for details

extracellular domain, which has been shown to occur in and promote metastasis of nasopharyngeal carcinoma cells [99]. In another study, PIK3CA overexpression in the mouse oral epithelium leads to increased tumor invasiveness and metastasis by inducing epithelial-to-mesenchymal transition (EMT). This study of PIK3CA-driven SCCHN emphasized the importance of 3-phosphoinositide-dependent protein kinase (PDK1) rather than AKT as a key effector [122]. Bozec and colleagues reported that the mTOR inhibitor temsirolimus in combination with cetuximab has a synergistic effect in NOD scid gamma (NSG) mice injected with SCCHN cells into the mouth floor. The combination of these two drugs significantly reduced tumor growth by inhibition of both the MAPK and the PI3K/AKT/mTOR pathway [123].

2.3.3.3 STAT

The signal transducers and activators of transcription (STAT) proteins were originally identified as downstream effectors of non-tyrosine kinase cytokine receptors, such as IL-6, IL-22, IFN-α/β, and IFN-λ. However, STATs can also be directly activated by EGFR, or by EGFR effectors such as c-Src [124], and constitutive activation of STATs has been reported in SCCHN [125]. Activated STATs migrate from cytoplasm to nucleus and upregulate the expression of many proteins associated with tumorigenesis, including the prosurvival factor NF-κB [126]. STAT family activation contributes to cancer cell survival and protects cells from apoptosis, which makes it a potentially useful therapeutic target [127], although there is some debate, as one study of a group of 102 SCCHN patients found nuclear STAT3 localization was associated with improved survival [128]. Additionally, Wheeler et al. reported that STAT3 activation can promote invasion of head and neck cancer cells bearing EGFRvIII and contributes to cetuximab resistance [129]. In vitro studies have shown that simultaneous inhibition of JAK1-STAT3 with JAK1i inhibitor and EGFR (cetuximab) in combination with radiation has a synergistic effect and leads to radiosensitization of human head and neck cancer cells and apoptosis [130]. Pre-irradiation inhibition of STAT5, STAT6, and MEK1/2 by 573,108, leflunomide, and U0126, respectively, in a panel of SCCHN cells, led to decreased survival following irradiation [131].

2.3.3.4 PLC/PKC

PLC is recruited by phosphorylated EGFR and subsequently activated. Primary tumors express elevated levels of total and phosphorylated PLCγ (one of six isotypes: β, γ, δ, ε, ζ, and η; [132]), and EGFR-stimulated activation of PLCγ promotes invasion of SCCHN [133]. PLCγ inhibition decreases the invasive potential of prostate, breast, and head and neck carcinoma cells [134, 135]. Once activated, PLC hydrolyzes PIP2 to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). DAG in turn activates members of the PKC family, which is composed of 12 different isoforms in mammals [18]. Protein kinase Cε [136] has been proposed as a promising prognostic factor for relapse and OS of SCCHN [137]. PKCζ is highly expressed in SCCHN tumors and mediates EGF-induced growth of SCCHN tumor cells by regulating MAPK [138]. A recent study demonstrated that resistance to PIK3CA inhibitors occurs due to the induction of the RTK AXL, which interacts with EGFR and activates PKC and mTOR, leading to cancer cell survival [139].

2.3.3.5 Src

Activation of members of the Src kinase family (Blk, Fgr, Fyn, Hck, Lck, Lyn, Src, Yes, and Yrk; [140]) by EGFR and ErbB2 positively regulates cell proliferation, migration, adhesion, and tumor angiogenesis, with activation seen in many cancer

types, including SCCHN [141–143]. In SCCHN, Src contributes to EGFR-dependent activation of STAT3 and STAT5, which, as mentioned above, are important for tumor growth [144]. Reciprocally, Src helps to activate EGFR by participating in G protein-coupled receptor-initiated TGFα release [145]. Changes in the interaction between Src and EGFR have been suggested to be involved in resistance to EGFR-targeting antibodies such as cetuximab by increasing translocation of EGFR to the nucleus (Sect. 2.2.2) [143, 146]. Src additionally interacts with other RTKs that are upregulated during acquisition of resistance to EGFRIs, such as IGF-1R (insulin-like growth factor-1 receptor) and others [147]. In gastric cancer, c-SRC-mediated activation of EGFR was shown to be induced by the receptor activator of NF-κB ligand (RANKL)/RANK pathway, promoting resistance to cetuximab [148]: whether this mechanism is relevant to SCCHN remains to be determined, although the fact that RANKL function has recently been found to be important for SCCHN progression is suggestive [149].

2.3.3.6 Nuclear Factor-κB (NF-κB)

High expression and constitutive activation of the transcription factor nuclear factor-κB (NF-κB) has been directly linked to tumorigenesis, metastasis, and chemoresistance in many cancers including SCCHN [150–154], with particularly high levels of NF-κB in highly metastatic cells [150, 155]. NF-κB induction of matrix metalloproteinases MMP-1, MMP-2, MMP-9, and MMP-14, fibronectin, ß1 integrin, and vascular endothelial growth factor C is strongly associated with tumor progression and metastasis [155]. In SCCHN, NF-κB activation has been described as both independent of and dependent on EGFR signaling [156–158]. In the EGFR-dependent activation of NF-κB, phosphorylated EGFR activates PI3K, ERK1/2, and STAT3, all of which are associated with increased NF-κB activity (Fig. 2.3) [156]. Depletion of NF-κB pathway components or pharmacological inhibition of NF-κB significantly increased cell death induced by erlotinib in EGFR-mutant lung cancer cells [159], supporting relevance of this signaling axis [160].

2.3.3.7 Cyclin D1

Cyclically induced expression of the cell cycle regulatory protein cyclin D1 (CCND1) promotes the key G1-to-S phase transition through formation of complexes with CDK4 and CDK6. The CCND1-CDK4/6 complex phosphorylates retinoblastoma tumor suppressor protein (Rb), inhibiting its activity and allowing changes in gene expression that promote S phase progression [161]. Analysis of SCCHN cell lines found common *CCND1* amplification and/or overexpression, which was associated with resistance to gefitinib [162]. In a group of 103 SCCHN

patients' tumor samples, *CCND1* amplification was observed in 30% (31/103) of patients and had a statistically significant association with recurrence, distant metastasis, and survival at 36 months [163]. EGFR expression coupled with CCND1 overexpression was found to be associated with sensitivity to combination therapy with CDK4/6 and ERBB-targeting inhibitors (palbociclib and lapatinib) in HPV(-) SCCHN cell models, while integrated analysis of CDK4/6, CCND1, and EGFR expression refined ability to predict SCCHN prognosis [164].

2.4 EGFR and Targeted Inhibitors

For patients with locally advanced SCCHN, randomized controlled trials have shown that the addition of chemotherapy to radiotherapy improves 3-year overall survival (51% for chemoradiotherapy compared to 31% for radiation alone) [165] and disease-free survival (37% for radiation plus chemoradiotherapy compared to 23% for radiation alone) [166], albeit at the cost of increased toxicity [167]. Targeting EGFR is now a well-established therapeutic strategy for SCCHN treatment [168], as EGFR inhibition seems to prevent activation of DNA repair mechanisms that enable cancer cells to survive radiation- or chemotherapy-induced DNA damage [169, 170]. However, the monotherapy response rate to cetuximab is only 10% in patients with platinum-refractory SCCHN [171]. It is unknown if this observation is due to cell-mediated immunity; data from R0522 suggests not, as cetuximab benefit did not correlate with FCγ subtype or markers of inflammation [172]. Alternatively, it may reflect the small proportion of cancers that are truly EGFR dependent. Numerous data suggest that high levels of EGFR may accelerate repopulation, a condition of enhanced cellular proliferation after exposure to ionizing radiation, contributing to the radioresistance associated with head and neck cancers [173, 174].

Despite potential secondary mechanisms, preclinical and clinical data support the premise that the inhibition of EGFR activity increases radio- and chemosensitivity of SSCHN tumors [173–175]. However, conflicting data regarding the sensitizing potential of EGFR inhibitors (EGFRIs) exist: for example, the RTOG 0522 phase III trial showed that the addition of the EGFRI cetuximab (Sect. 2.4.1.1) does not improve PFS rates to chemoradiation in patients with stage III and IV SCCHN, potentially due to overlapping radiosensitization properties of cisplatin and cetuximab treatment [176]. Newer agents that target EGFR remain under investigation (e.g., NCT02555644, NCT01427478, NCT00588770, and others: see also Table 2.1). Further, the side effect profiles of EGFRIs have been generally favorable compared to standard chemotherapeutics [177–179].

2.4.1 Monoclonal Antibodies Targeting EGFR and Other ErbB Proteins

Cetuximab, a monoclonal antibody targeting EGFR, plays a significant role in the treatment of SCCHN. Cetuximab, the pioneer for antibody-based anti-ErbB therapy in SCCHN, was approved for treatment of locally or regionally advanced SCCHN in 2006 [175] and for metastatic SCCHN in 2011 [180]. While cetuximab represents the EGFR inhibitor with the most clinical data and the most significant results in the treatment of SCCHN, multiple additional antibodies targeting ErbB receptors are currently being investigated in clinical trials. Promising results of EGFR-targeting antibodies used in mice were first published in 1984 [181], over two decades before cetuximab was approved for clinical use. This section introduces several relevant antibodies and covers their current status as it pertains to SCCHN, with some additional information found in Table 2.1.

2.4.1.1 Cetuximab

Cetuximab is a chimeric monoclonal antibody that inhibits EGFR by binding to its extracellular domain (Fig. 2.4). Cetuximab binds to EGFR with a higher affinity than its natural ligands EGF and TGFα [182–184]. Once bound to the EGFR extracellular domain, cetuximab occludes the ligand-binding site, thus inhibiting ligand-dependent EGFR signaling [185]. Depletion of the targeted receptors from the cell surface via downregulation is a second mechanism of effective EGFR inhibition [186]. Additionally, binding of cetuximab to EGFR enhances antibody-dependent, cell-mediated cytotoxicity via natural killer cells and macrophages in model systems [187, 188]. Cetuximab has been approved for three indications in patients with SCCHN. These are patients with locally or regionally advanced SCCHN (cetuximab in combination with radiation therapy; [175]), patients with recurrent or metastatic platinum-refractory SCCHN (cetuximab monotherapy; [189]), and patients with recurrent locoregional and/or metastatic SCCHN not refractory to platinum-based therapies (cetuximab in combination with platinum chemotherapy and 5-fluorouracil as first-line therapy; [180]).

Phase I studies of cetuximab defined the dose and schedule required to maintain biologically active and tolerable levels [190, 191]. Whether used in combination with chemotherapy or radiotherapy, or as monotherapy, cetuximab was found to have nonlinear saturation kinetics. Median serum cetuximab terminal half-life ranged from 14 to 97 h with doses from 5 to 300 mg/m2. Skin reactions increased significantly at doses of 500 mg/m^2 or higher. Given the results of these phase I trials, the recommended cetuximab regimen was established as an initial loading dose of 400 mg/m^2 followed by weekly doses of 250 mg/m^2 [190, 191].

The landmark phase III study of cetuximab added to radiation published by Bonner and colleagues led to the approval of cetuximab for the treatment of patients with locally or regionally advanced SCCHN [175]. Four hundred and twenty-four

Table 2.1 Monoclonal antibody and small-molecule inhibitors of EGFR

Monoclonal antibodies				
Name	*Target*	*Stage of development/ trials*	*Administration*	*Comments*
Cetuximab	EGFR; extracellular; domain III	Approved for use in SCCHN/ EXTREME	IV, weekly	First targeted therapy for SCCHN [175, 180]. In combination with radiotherapy or cisplatin significantly increases progression-free survival [175, 192, 195]. Mediates antibody-dependent cellular cytotoxicity (ADCC) [198]
Panitumumab	EGFR; extracellular; domain III	Phase III/ PRISM, PARTNER, SPECTRUM (SCCHN)	IV, every 3 weeks	Potentially less immunogenic than chimeric mAbs; low rate of infusion-related hypersensitivity reaction [199]. In a combination with cisplatin is tolerable and demonstrates improved clinical outcome for high-risk, resected, HPV-negative SCCHN patients [357]. A phase I study of panitumumab, carboplatin, paclitaxel, and radiation for locally advanced disease has indicated that this combination is feasible with tolerable toxicity, and 69% of patients had a complete response and 34% had a partial response [201]
Zalutumumab	EGFR; extracellular; domain III	Phase III, DAHANCA 19 (SCCHN)	IV, every 2 weeks	Particularly effective induction of ADCC. In 286 patients with metastatic/recurrent SCCHN after failure of platinum-based therapy, zalutumumab plus best supportive care was compared with best supportive care plus methotrexate. Zalutumumab did not increase OS, although PFS was extended [358]
Nimotuzumab	EGFR; extracellular; domain III	Phase I/II (SCCHN)	IV, weekly	Binds with less affinity than cetuximab; mild to no skin toxicity. In a double-blind trial, patients with unresectable locoregional SCCHN were assigned to receive first-line therapy with nimotuzumab plus radiotherapy versus placebo plus radiotherapy. Complete response rates were significantly better in the nimotuzumab group with 59.5% for patients receiving nimotuzumab and radiotherapy versus 34.2% of patients receiving radiotherapy alone [359]. Nimotuzumab was found to be safe and well tolerated in a group of 92 treatment-naïve SCCHN patients and led to a benefit to long-term survival in combination with chemoradiotherapy (CRT) [360]

(continued)

Table 2.1 (continued)

Matuzumab	EGFR; extracellular domain III	No active trials	N/A		Binds at a completely different epitope than cetuximab [208]. Matuzumab has been evaluated in a phase I dose escalation study focused on patients with advanced EGFR-positive esophagogastric cancer. Matuzumab in combination with epirubicin, cisplatin, and capecitabine (ECX) was well tolerated. furthermore, in skin biopsies, decreased phosphorylation of EGFR and MAPK was detected [361]. Surprisingly, the phase II study of matuzumab in combination with ECX did not increase response or survival in patients with metastatic esophagogastric cancer [362]
XGFR*	EGFR; IGF-1R	No active trials	N/A		Binds EGFR and insulin growth factor receptor 1 (IGF-1R), which was shown to overcome resistance to EGFR inhibitors [210]. XGFR* is more selective for IGF-1R binding due to affinity maturation and stability to oxidative and thermal stress. In addition, the XGFR* induces ADCC. Showed a significant effect on different cancer types, including lung and pancreatic, in vivo and in vitro [211]
Tyrosine kinase inhibitors					
Gefitinib	ATP binding site; intracellular; TK domain of EGFR; reversible binding	Phase III		PO, daily	First TKI to reach a phase III investigation; trial failures led to its withdrawal from clinical investigation in SCCHN
Erlotinib	ATP binding site; intracellular; TK domain of EGFR; reversible binding	Phase II and phase III studies		PO, daily	Investigated as first-line treatment with radiotherapy or chemoradiotherapy in locally advanced SCCHN. Erlotinib has been evaluated in multiple phase II and phase III studies including studies focused on erlotinib combined with cetuximab, carboplatin, and paclitaxel (NCT01316757), docetaxel and radiation therapy (NCT00720304), docetaxel and cisplatin or carboplatin (NCT01064479), and cisplatin (NCT00410826) [168]. In patients with locally advanced SCCHN, the addition of erlotinib to standard cisplatin-radiotherapy regimens has not improved to therapeutic efficacy (CRR or PFS) [363]. However, a phase II clinical study of combination of erlotinib and docetaxel with intensity-modulated radiotherapy (IMRT) for locally advanced SCCHN suggested the inclusion of erlotinib led to a significantly better outcome [364]

(second generation)	site; intracellular; TK domain of EGFR and ErbB2/ HER2; reversible binding		daily	dual-action small-molecule drug as an adjuvant to postoperative chemoradiation in SCCHN patients (e.g., NCT00387127), although addition of lapatinib to standard therapy RT/CDDP was found not to extend DFS [250, 365]. In general developments with this reagent, a novel type of lapatinib delivery, using hybrid nanoparticles, demonstrated a great potential in enhanced therapeutic effect in breast tumors and could be potentially applied for the treatment of SCCHN [366]
Afatinib (second generation)	ATP binding site; intracellular; TK domain of EGFR, ErbB2, ErbB4; irreversible binding	Phase III	PO, daily	Multi-specificity, comparable outcome to cetuximab monotherapy in a randomized phase II study. Phase III clinical trial has demonstrated that afatinib treatment significantly improved PFS and had a manageable safety profile in a group of 322 SCCHN patients in comparison with methotrexate (NCT01345682) [367]. In another ongoing phase III study, a group of patients with SCCHN after postoperative radiochemotherapy was randomized to afatinib or placebo for 18 months in a phase III trial designed to detect an improvement in PFS (NCT01427478)
Dacomitinib (second generation)	ATP binding site; intracellular; TK domain of EGFR, ErbB2, ErbB4; irreversible binding	Phase I and phase II	PO, daily	Has an activity against wild-type and mutant receptors, including EGFRvIII. Compared to phase II studies involving gefitinib or erlotinib, dacomitinib produces favorable outcomes in terms of disease control and survival [368]. In another clinical trial, dacomitinib demonstrated clinical efficacy with manageable toxicity in R/M-SCCHN patients who had failed prior treatment with platinum agents [369]

(continued)

Table 2.1 (continued)

CUDC-101	ATP binding site; intracellular; TK domain of EGFR and ErbB2; also histone deacetylase (HDAC)	Phase I	IV, 3 times per week	Structurally contains elements of TKIs (e.g., erlotinib) and elements of HDAC inhibitors (e.g., vorinostat). In a phase I clinical trial, CUDC-101 in combination with cisplatin and radiation was well tolerated. further patients' biopsies analysis demonstrated an inhibition of EGFR [264]
TX1-121-1	Binds the unique ErbB3 ATP-binding site (Cys721)	No active trials	N/A	Showed no significant effect against ErbB3-dependent signaling and growth in vitro. Subsequent conjugation of TX1-85-1 with adamantane (called TX2-121-1) caused partial proteolytic degradation of ErbB3 and inhibited ErbB3/ErbB2 and ErbB3/c-met heterodimerization in vitro [265]

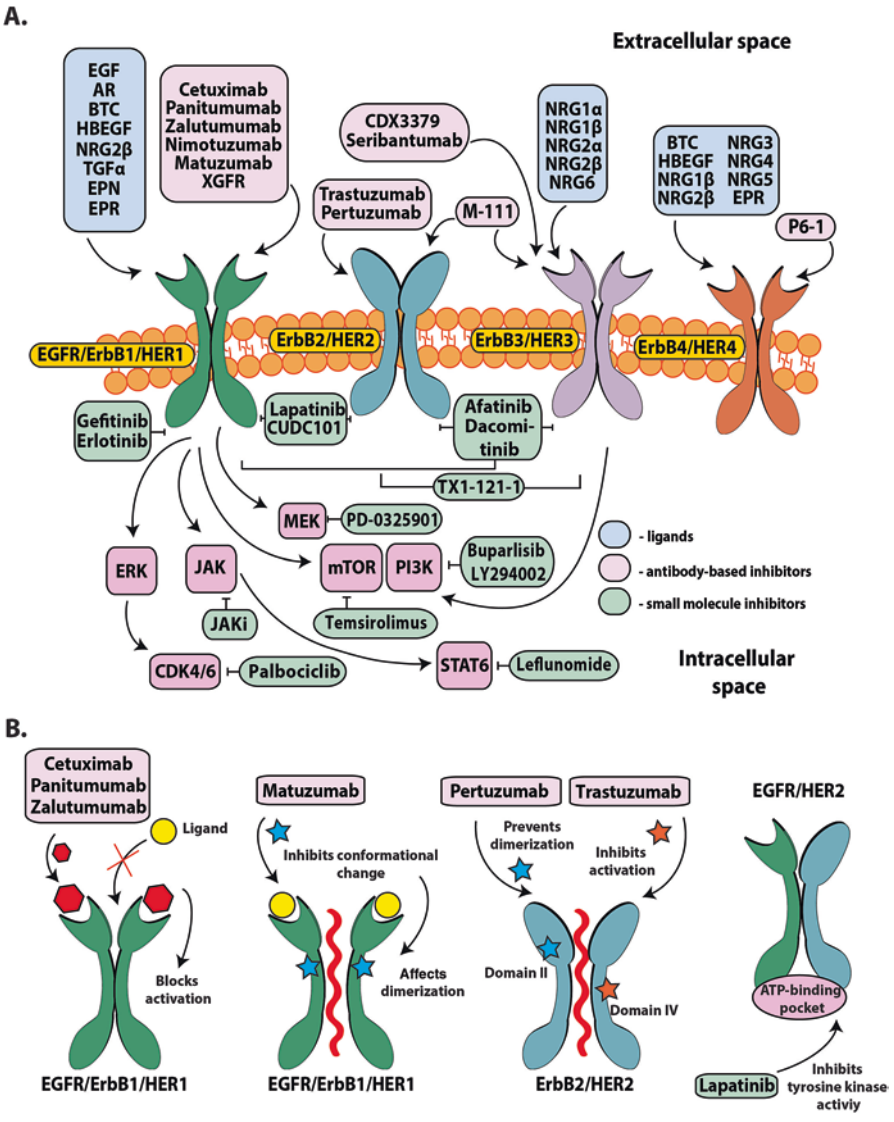


Fig. 2.4 Activators and inhibitors of the ErbB proteins and critical signaling effectors. (**a**) *Blue* boxes indicate activating ligands for the indicated ErbB dimers. *Pink* boxes indicate monoclonal antibodies targeting ErbB family proteins. *Green* boxes indicate small-molecule inhibitors of ErbB proteins or specific signaling effectors. (**b**) Mechanism of action for selected ErbB family inhibitors

patients undergoing definitive treatment with radiation were randomized to radiotherapy alone or to radiotherapy plus cetuximab. Cetuximab and radiotherapy significantly improved median OS and median progression-free survival (PFS) when compared to radiation alone [175]. Long-term follow-up of the Bonner study showed an absolute survival increase of 9% in the treatment group receiving cetuximab in combination with radiation therapy (5-year survival of 45.6% for cetuximab/radiation vs. 36.4% radiation alone) [192].

In the case of patients with platinum-refractory recurrent or metastatic SCCHN, Trigo et al. observed an overall response rate to cetuximab in combination with platinum-based chemotherapy of 10% and an overall survival of 183 days in a single-arm study of 96 patients with platinum-refractory recurrent disease [193]. The observed response and survival rate were similar to rates expected with platinum therapy alone in chemotherapy-naïve patients, supporting combination cetuximab and chemotherapy [194].

In a phase III randomized trial, Burtness and investigators from the Eastern Cooperative Oncology Group compared the impact of cisplatin plus a placebo with the impact of cisplatin plus cetuximab in previously untreated patients with recurrent and metastatic SCCHN. This study demonstrated that the addition of cetuximab significantly increased the objective response rate (26% response rate for cisplatin/cetuximab and 10% response rate for cisplatin/placebo; $p = 0.03$). PFS increased from 2.7 to 4.2 months; this difference was not statistically significant, in a trial which was underpowered because the statistical assumptions underestimated PFS in the control arm. Unexpectedly, Burtness et al. also observed that cetuximab was not active in patients with the highest EGFR staining density and intensity. It was hypothesized that several factors may have contributed to this observation: the small sample size ($n = 123$), suboptimal cetuximab dosing for cases of high-density EGFR occurrence, stochastic interactions at high EGFR density, or constitutive downstream signaling not accounted for in the study [195]. Indeed, subsequent investigators have identified loss of PTEN expression as predictive of resistance to EGFR inhibition in SCCHN, as will be detailed below.

Vermorken et al. built on these findings to conduct a phase III clinical trial (EXTREME trial) investigating the efficacy and safety of platinum, fluorouracil (5-FU), and cetuximab as first-line treatment of recurrent and metastatic SCCHN in 442 patients. The EXTREME phase III trial randomly assigned patients to receive cisplatin or carboplatin plus 5-FU and cetuximab or platinum plus 5-FU alone. Six cycles of chemotherapy were the limit for both arms of the study; however, cetuximab was continued until disease progression or prohibitive toxicity. The addition of cetuximab to chemotherapy significantly increased median OS (10.1 months in the cetuximab group and 7.4 months in the chemotherapy-alone group; $p = 0.04$) and PFS (5.6 months in the cetuximab group to 3.3 months in the chemotherapy-alone group; $p < 0.001$) when compared to standard chemotherapy alone [180]. Importantly, additional analysis of the EXTREME data provided further evidence that, in the case of SCCHN, EGFR expression level is not a clinically useful predictive biomarker [196]. Lei et al. demonstrated that resistance to cetuximab occurs in part through autophagy activation involving NLRX1-TUFM protein complex.

Analysis of patients' tumor specimens also confirmed that cetuximab therapy leads to the increased expression of autophagy SQSTM1/p62 protein, and data analysis from clinical trials showed a positive correlation between cetuximab-induced autophagy and poor prognosis [197].

2.4.1.2 Additional EGFR-Targeting Antibodies

Panitumumab is a fully humanized immunoglobulin IgG2 monoclonal antibody that, like cetuximab, binds to EGFR domain III and, in the process, inhibits EGF and TGFα binding [185] (Fig. 2.4). In contrast to cetuximab [198], panitumumab does not mediate antibody-dependent cellular cytotoxicity and has been shown to have a very low rate of infusion-related hypersensitivity reaction [199]. Another study found that panitumumab effectively inhibits EGFR signaling, but this antibody had a reduced ability to enhance dendritic cell maturation, in comparison with cetuximab, a finding of uncertain clinical significance [200] [201]. The SPECTRUM trial (phase III; NCT00460265) compared cisplatin/5-FU plus panitumumab to cisplatin/5-FU alone in patients with metastatic/recurrent SCCHN. The addition of panitumumab to chemotherapy did not significantly improve median OS versus chemotherapy alone but did improve median PFS (5.8 vs. 4.6 months) [184, 202]. Another study comparing PFS in patients with locally advanced (LA) SCCHN treated with standard-fractionation RT plus high-dose cisplatin versus accelerated-fractionation RT plus panitumumab demonstrated no difference between these two treatment types [203], providing further evidence that panitumumab has activity in SCCHN.

Zalutumumab is a human IgG1 high-affinity antibody also targeting EGFR domain III, and, just like panitumumab and cetuximab, zalutumumab is thought to block ligand binding, but with exceptional tumor specificity at lower doses [185] (Fig. 2.4). A phase III trial in metastatic or recurrent SCCHN following platinum failure compared zalutumumab to best supportive care, defined to include methotrexate monotherapy. Median overall survival was modestly increased by zalutumumab (hazard ratio [HR] for death, stratified by performance status, was 0.77; unadjusted $p = 0.0648$). Progression-free survival was significantly longer in the zalutumumab group (HR for progression or death was 0.63, 95% CI 0.47–0.84; $p = 0.0012$). A phase III trial is currently underway (DAHANCA 19; NCT00496652) to determine if the addition of zalutumumab to radiotherapy improves locoregional control. Preliminary results from this trial did not demonstrate beneficial effect from addition of zalutumumab [204].

Nimotuzumab has been approved for SCCHN in several countries, not including the USA. Nimotuzumab is a humanized murine IgG1 monoclonal antibody that also blocks interaction between ligand and receptor by binding to EGFR domain III, but with lesser affinity than some of the other antibodies [205]. The therapeutic implications of this reduced affinity are unclear, but nimotuzumab has been shown to have mild to absent skin toxicity, eliminating a clinically important adverse effect commonly associated with cetuximab [206]. An early pharmacodynamic study

showed nimotuzumab plus radiotherapy was tolerated with no evidence of skin rash in patients with unresectable SCCHN [207]. Hence, nimotuzumab may offer an EGFR-targeted therapy with a favorable side effect profile. The possibility of combining high- and low-affinity antibodies to optimize antibody penetration in larger tumors has not been explored clinically.

Matuzumab, another humanized mouse monoclonal antibody, also binds to EGFR domain III, but at a completely different epitope than the previously mentioned antibodies (Fig. 2.4). This was confirmed by experiments in which cetuximab and matuzumab were observed to simultaneously bind to EGFR [208]. When bound to EGFR, matuzumab was determined to predominantly prevent domain II from assuming the configuration, in relation to domain III, necessary for high-affinity ligand binding [185], interrupting EGFR signaling. Matuzumab has not been tested in SCCHN.

XGFR*. In 2014, Schanzer et al. developed a novel one-arm single-chain Fab heterodimeric bispecific IgG (OAscFab-IgG; XGFR*) antibody, which targets both the insulin-like growth factor receptor type I (IGF-1R) and EGFR; importantly, this antibody has only one binding site for each target antigen [209]. It was previously shown that signaling through IGF-1R can overcome resistance to EGFR inhibitors, and EGFR-dependent signaling can confer resistance to IGF-1R inhibitors [210]. In addition, the XGFR* antibody has an afucosylated Fc portion and induces antibody-dependent cell-mediated cytotoxicity (ADCC). Thus, inhibition of both these receptors by XGFR* antibody had a significant effect on different cancer types, including lung and pancreatic, in vivo and in vitro [211]. Clinical evaluation of this agent is required.

2.4.1.3 Monoclonal Antibodies Targeting Other ErbB Proteins

Given the heterodimerization of EGFR with other ErbB family proteins, and the fact that overexpression of some of these proteins can compensate for EGFR inhibition during development of therapeutic resistance, a natural development has been to explore inhibition of additional EGFR family members in SCCHN [212–214].

Pertuzumab binds the ErbB2 dimerization domain and blocks its interaction with all four ErbB family members [215] (Fig. 2.4). Erjala et al. observed that increased expression levels of phosphorylated ErbB2 and total ErbB3 were associated with SCCHN cell line resistance to gefitinib [216]. Confirming the importance of ErbB2 in resistance, when gefitinib was combined with pertuzumab, significant growth inhibition of relatively gefitinib-resistant SCCHN cell lines was observed. Phosphorylated ErbB2 and total ErbB3 were not predictive of resistance to cetuximab [216].

Seribantumab (SAR256212, MM-121), targeting ErbB3, has been shown to be effective by inhibiting ligand-induced ErbB3 signaling [50, 217]. In another study, seribantumab demonstrated antitumor activity in breast cancer cell models [218]. More recently, in a randomized phase II trial of seribantumab in combination with weekly paclitaxel compared with paclitaxel alone, the combination showed no effect in PFS among 140 patients with ovarian cancer [219].

MM-111 is a bispecific single-chain antibody that simultaneously targets ErbB2 and ErbB3. An antitumor effect of MM-111 was shown in several in vivo cancer models [220].

P6-1. Recently, a new monoclonal antibody that specifically targets neuregulin-1-induced ErbB4 activation was developed. P6-1 was tested on breast cancer cell lines and showed moderate anticancer activity [221].

Trastuzumab. The ErbB2/HER2-targeting antibody trastuzumab is an invaluable drug for breast cancer and other epithelial tumors [222–225]. In vitro studies have shown that trastuzumab enhances the efficacy of gefitinib [226] and cetuximab [227] in SCCHN cells. Surprisingly, analysis of the mRNA expression of EGFR and ErbB2 indicated lack of correlation with efficacy of the combination therapy [227]. Moreover, an independent study found that a subset of non-ErbB2-amplified SCCHN cells was nevertheless extremely responsive to the small molecule multi-ErbB inhibitor lapatinib, based on activation of a neuregulin-ErbB3 loop [228]. A number of studies have been investigating factors contributing to trastuzumab. Kulkarni and colleagues found that the Ca^{2+}-activated Cl^- channel TMEM16A significantly contributes to tumor growth in SCCHN and some other tumors, with levels of TMEM1A increased in trastuzumab-resistant cells. They also found that concurrent treatment of these cells with cetuximab and TMEM16A inhibitor led to cell death, demonstrating a novel role of TMEM16A in regulation of the EGFR and HER2 pathways [229].

Duligotuzumab (MEHD7945A) blocks ligand binding to EGFR and HER3 and may contribute to antibody-dependent cell-mediated cytotoxicity (ADCC) in cells overexpressing these proteins. A randomized phase II study (MEHGAN, NCT01577173) evaluated drug efficacy in patients with recurrent/metastatic (R/M) SCCHN, treated with duligotuzumab or cetuximab. In this study, duligotuzumab demonstrated a higher efficacy than cetuximab only in tumors with greater expression of NRG1, a ligand for ERBB3 and ERBB4 [230]. Duligotuzumab in combination with cisplatin/5-fluorouracil or carboplatin/paclitaxel demonstrated a promising effect in patients with recurrent/metastatic SCCHN [231]. An independent study performed in a group of SCCHN and colorectal cancer patients demonstrated similar results, with duligotuzumab as a single agent possessing pronounced antitumor activity [232].

CDX3379 (formerly KTN3379) locks HER3 in an inactive confirmation by docking to its extracellular domain, effectively promoting inhibition of both ligand-dependent or ligand-independent activation of HER3 [233]. CDX3379 was found to be efficient against HPV-positive SCCHN cell lines and PDXs [234]. CDX3379 is currently being evaluated for safety in a phase I clinical trial (NCT02014909).

2.4.2 Tyrosine Kinase Inhibitors (TKIs) Targeting EGFR and Other ErbB Proteins

TKIs block EGFR activation by inhibiting the cytoplasmic tyrosine kinase domain and have proven valuable agents in a number of cancer types. First-generation TKIs for EGFR, including gefitinib and erlotinib, reversibly bind the ATP-binding pocket

of the kinase domain and are EGFR specific. Second-generation TKIs relevant to SCCHN, including lapatinib, afatinib, and dacomitinib, target multiple ErbB members (in the case of afatinib and dacomitinib, irreversibly) (Table 2.1; Fig. 2.4) [168, 182].

Gefitinib, an orally administered, small-molecule, reversible EGFR TKI, was the first TKI to reach phase III trials; however, overall results have dampened expectations. Early studies suggested a clinical benefit of gefitinib similar to cetuximab [235, 236]; unfortunately, more recent results do not indicate a significant role for gefitinib in the management of SCCHN [168, 237, 238]. In treatment-refractory SCCHN, gefitinib did not improve OS when compared to methotrexate [239]. These findings were consistent when gefitinib was administered orally at 250 or 500 mg daily, despite the fact that single-arm studies had demonstrated favorable response rates when gefitinib was administered at 500 mg/day [235, 239]. The maximum tolerated dose of gefitinib has been established to be 600 mg/day, with dose-limiting toxicity observed at 1000 mg/day [240]. In a phase II study of 44 patients with SCCHN, Perez et al. investigated if doses higher than 500 mg/day would produce increased skin toxicity, which is thought to be associated with improved response to EGFRIs. Patients treated with 750 mg/day had increased incidence of skin toxicity compared with patients receiving 500 mg/day (58 and 9% grade 2 skin toxicity for 750 and 500 mg/day, respectively); however, the higher dose of gefitinib failed to significantly improve outcome [241]. Gefitinib (250 mg/day) in combination with docetaxel (docetaxel/gefitinib vs. docetaxel alone) was evaluated in a phase III trial of patients with metastatic or locally recurrent SCCHN. In this study, Argiris et al. were unable to demonstrate a statistically significant survival benefit for patients receiving the docetaxel/gefitinib combination. However, subgroup analysis demonstrated that for patients younger than 65 years of age, the addition of gefitinib to docetaxel did increase survival significantly (median survival of 7.6 vs. 5.2 months; $p = 0.04$) [242]. In the case of NSCLC, non-smoking patients had a significantly better response to gefitinib treatment and demonstrated prolonged PFS in comparison with smoking patients [243]; this may be related to the fact that smoking induces detoxification enzymes such as cytochromes (CYP2D6, CYP1A2) that contribute to the metabolism of gefitinib and erlotinib [244], limiting their efficacy. It is important to note that gefitinib therapy demonstrates high interpatient variability in plasma levels even in healthy male volunteers, varying up to 15-fold between individuals [245], suggesting the need for further studies to allow more accurate individual dosing with gefitinib and other 4-anilinoquinazolines (including erlotinib and lapatinib).

Erlotinib, the second first-generation TKI, like gefitinib, is an orally administered, small-molecule, reversible TKI. In 115 patients with refractory SCCHN, erlotinib led to disease stabilization in 38.3% of patients for a median duration of 16.1 weeks. The median PFS was 9.6 weeks and the median OS was 6.0 months [246]. Smoking status of lung cancer patients was also found to be an important determinant of the pharmacokinetics of erlotinib during therapy, leading to a 2.8-fold increased C_{max} and decreased metabolic clearance of erlotinib in non-smokers to compare with smoking patients [247].

Lapatinib has dual specificity, targeting EGFR and ErbB2 [248] (Fig. 2.4). Surprisingly, in a phase II study focused on recurrent/metastatic SCCHN, lapatinib, as monotherapy, failed to lead to objective responses. On-treatment biopsies were performed: although ErbB2 levels were significantly decreased, EGFR phosphorylation remained unaffected in these specimens [249]. Addition of lapatinib to standard therapy radiation and cisplatin": standard therapy with radiation and cisplatin was found not to extend DFS [250]. Another clinical trial focusing on the combination of lapatinib with capecitabine in patients with metastatic SCCHN (NCT01044433) is still in process. Additional phase II trials are investigating the effect of lapatinib in combination with definitive chemoradiation followed by 1 year of lapatinib maintenance for locally advanced (LA) SCCHN (NCT00387127) and definitive radiation for the patients who had low tolerance to CRT (NCT00490061) [251].

Afatinib, also a multi-specific TKI, irreversibly targets three of the four ErbB family members: EGFR, ErbB2, and ErbB4 [252]. In a comparison to cetuximab, afatinib showed similar antitumor activity in patients with recurrent or metastatic SCCHN after failing platinum therapy. Median PFS was 15.9 weeks with afatinib and 15.1 weeks with cetuximab [253]. In another clinical trial, 2 weeks of pretreatment of newly diagnosed SCCHN patients with afatinib led to a better metabolic FDG-PET profile than seen in patients receiving no treatment (NCT01538381) [254]. Based on the results from a group of R/M SCCHN patients, Cohen et al. established potential biomarkers of afatinib clinical outcome. Prolonged PFS after afatinib treatment was associated with amplified *EGFR*, negative p16 status, high expression of PTEN, and low expression of HER3 [255]. According to the most recent data, afatinib treatment of 411 SCCHN patients with complete response to chemoradiation did not demonstrate any improvement in disease-free survival to compare with placebo group (NCT01345669) [256].

Dacomitinib, like afatinib, is an irreversible inhibitor of EGFR, ErB2, and ErbB4 [257]. Dacomitinib is a potent inhibitor of wild-type EGFR as well as EGFR with activating mutations. Furthermore, dacomitinib appears to be active against the *T790 M* secondary *EGFR* mutation, which generally renders cancer cells resistant to erlotinib and gefitinib, in NSCLC [258]. Dacomitinib reduced the viability of SCCHN cells and in combination with ionizing radiation (IR) more effectively delayed tumor growth in vivo in a dose-dependent manner [259]. A phase I clinical trial investigating the efficacy of dacomitinib plus chemotherapy in treating SCCHN was completed (NCT01737008), but the results are not yet published. In another study (NCT01484847), dacomitinib was administered via gastrostomy feeding tube (GT) to evaluate the efficacy of this method. The results have demonstrated that the pharmacokinetics of dacomitinib administered by GT are significantly decreased to compare with the oral administration [260]. In a completed phase II trial (NCT00768664) of 69 patients with recurrent/metastatic SCCHN treated with dacomitinib, pretreatment tumor and normal tissue specimens were analyzed. No biomarkers of dacomitinib treatment efficacy were identified. Nevertheless, dacomitinib treatment was associated with increased OS in HPV-driven cancers [261].

CUDC-101 is a multi-targeted hybrid anticancer drug candidate with a complex mode of action. CUDC101 effectively inhibits EGFR, HER2, and histone deacetylase (HDAC) and has shown impressive activity in in vitro as well as in vivo cancer models [262, 263]. This hybrid inhibitor has been investigated in combination with cisplatin and radiation therapy in patients with locally advanced head and neck cancer as part of a phase I drug escalation trial (NCT01384799). The results of this study established the maximum tolerated dose (MTD) of CUDC-101 in combination with cisplatin and radiation. Further analysis of tumor biopsies demonstrated inhibition of EGFR [264].

TX1-121-1. ErbB3 plays an important role in the EGFR signaling through activation of EGF receptor [9]. The small-molecule agent TX1-85-1 covalently binds the unique ErbB3 ATP-binding site (Cys721). However, this compound showed no activity against ErbB3-dependent signaling and growth [265].

2.4.3 Targeting Critical EGFR/ErbB Effectors

A classic means by which cancer cells overcome resistance to inhibition of upstream activating oncogenes is to upregulate or activate one or more essential effectors operating downstream. A reactive therapeutic approach is to inhibit these downstream effectors, sometimes in combination with agents targeting the upstream oncogenic driver. In SCCHN, resistance to inhibitors of ErbB proteins has been associated with upregulation or activation of a number of downstream or lateral signaling effectors, including PI3K, MEK, ALK, and others. Some recent studies have evaluated inhibition of these targets in SCCHN.

Using xenograft experiments, Mizrachi et al. found that the inhibitor BYL719, which targets PI3Kα (p110α subunit of PI3K), is effective against SCCHN. Furthermore, they demonstrated that encapsulation of BYL719 into P-selectin-targeted nanoparticles leads to an accumulation of BYL719 in tumors, which promotes more effective inhibition of tumor growth and ameliorates side effects associated with BYL719 treatment [266]. Several isoform-specific PI3K inhibitors are under development and in clinical trials [267]. In preclinical studies, the specific PI3KCA inhibitor GDC-0032 was effective in controlling SCCHN in vitro and in vivo [267]. Co-targeting of EGFR and PI3K with erlotinib and BKM20, respectively, had synergistic antitumor effects and apoptosis induction in a panel of SCCHN cell lines and xenograft models of SCCHN.

There is some evidence that, in several types of cancer, including breast, non-small cell lung, and glioblastoma, inhibition of PI3K or mTOR can abrogate cell resistance to anticancer therapy [268]. Apitolisib (GDC-0980) is a dual inhibitor of class I PI3Ks and mTOR kinases. A phase I clinical trial of apitolisib indicated durable antitumor activity in a group of patients with solid tumors, including SCCHN [269]. This may predominantly reflect inhibitory activity against PI3K, as treatment of recurrent/metastatic SCCHN patients with the mTOR inhibitor everolimus showed no response to the therapy [270] [271]. However, other studies have

concluded that concurrent targeting of EGFR and PI3K is synergistic specifically because of inhibition of both axes of the AKT-mTOR pathway, coupled with translational regulation of anti-apoptotic Bcl-2 proteins [271].

Activation of ErbB kinases signals through RAS and RAF to activate downstream MEK/ERK kinases; RAS, PI3K, and other ErbB effectors activate AKT. Cetuximab inhibits ERK and AKT phosphorylation in cetuximab-sensitive SCCHN cells, whereas the level of AKT phosphorylation is unmodified in cetuximab-resistant cells [272]. Mohan et al. demonstrated that inhibition of MEK by PD-0325901 overcame resistance to the PI3K/mTOR inhibitor PF-5212384 and had a potential antitumor effect in SCCHN [273]. Increased activity of the anaplastic lymphoma kinase (ALK) was observed in late-stage human OSCC tumors and invasive OSCC cell lines. Both in vitro and in xenografts, concurrent inhibition of ALK (using the TAE684 inhibitor) and EGFR (with gefitinib) significantly reduced OSCC cell proliferation and tumor volume in comparison with ALK inhibition alone. Dual inhibition was associated with complete abolishment of AKT activation, whereas separate inhibition of EGFR or ALK only reduced it [274].

A study of esophageal squamous cell carcinoma showed that tumor cells treated with afatinib and erlotinib become rapidly resistant due to reactivation of the MEK/ERK pathway. This can be delayed by initial dual treatment with the MEK inhibitor trametinib used in combination with an EGFR inhibitor, which decreased tumor cell proliferation and survival [275]. Such strategies may be useful for SCCHN. The same study also noted the potential utility of combining CDK4/6 inhibitors with EGFR inhibitors in *EGFR*-amplified tumors [275]. At present, the CDK4/6 inhibitor palbociclib is in a phase I clinical trial in a complex with cetuximab for SCCHN patients. Preliminary results have demonstrated the safety of palbociclib with cetuximab in patients with recurrent/metastatic SCCHN. Encouragingly, tumor responses were observed, even in cetuximab- or platinum-resistant disease [276].

Checkpoint kinases 1 and 2 (Chk1/2) are critical regulators of the DNA damage response and important for regulation of cell cycle arrest in S phase to allow repair. In preclinical studies, simultaneous treatment with the Chk1/2 inhibitor prexasertib, cetuximab, and irradiation significantly decreased cell proliferation and survival of SCCHN cells both in vitro and in vivo. A clinical trial to test this treatment for patients with SCCHN is ongoing (NCT02555644) [277]. Huang et al. have performed whole-genome sequencing of cisplatin-resistant SCCHN tumors to find potential predictive biomarkers of dacomitinib resistance and identified a "platinum" mutational signature, involving a number of genes, including *REV3L*, which encodes the catalytic subunit of DNA polymerase ζ. Further investigations have demonstrated that depletion of REV3L dramatically enhanced the sensitivity of SCCHN cells to the ErbB2 inhibitor dacomitinib through translesion synthesis and homologous recombination [278].

FGFR1 was identified as a prognostic marker of SCCHN, and shown to be highly expressed in 82% (36/44) of HPV(+) and 75% (294/392) of HPV(-) SCCHN samples, and associated with poor OS and DFS in samples with HPV(-) status. Mechanisms of resistance to the FGFR inhibitor AZD4547 occur due to compensatory EGFR signaling [279], suggesting potential synergy in combining EGFR- and FGFR-targeting agents.

2.4.4 EGFR/ErbB2 and Immunotherapy/Immune Response

The past 5 years have seen the emergence of several distinct classes of immunotherapies as potent anticancer treatments. Some immunotherapies can be used to potentiate natural killer (NK) cell-mediated antibody-dependent cellular cytotoxicity (ADCC) against antibody-coated tumor cells, enhancing the antitumor activity of monoclonal antibodies [280]. Of specific relevance to EGFR inhibition, therapy with cetuximab mediates NK cell/dendritic cell (DC) cross talk by cross-linking FcγRIIIa. NK cells activated by cetuximab can upregulate the costimulatory receptor CD137 (4-1BB), which enhances NK cell activity upon treatment with urelumab (CB137 agonist). In a phase Ib trial, concurrent treatment of SCCHN patients with cetuximab and urelumab showed modulation of immune biomarkers and better survival of activated NK cells taking part in antitumor cell immunity [281]. Another group showed that NK cell-mediated ADCC increases upon stimulation with IL-12 in the presence of cetuximab-treated SCCHN cell lines. Combination IL-12 and cetuximab also significantly reduced tumor volume in a group of mice injected with a squamous cell carcinoma of tongue cell line (Cal-27). These results suggest that concurrent treatment with cytokines and cetuximab might have a beneficial effect in SCCHN therapy [282]. Kumai et al. have exploited the fact that HER3 expression is significantly increased upon EGFR inhibition therapy in SCCHN. They have used a HER3 peptide analog as a helper epitope for antigen presentation and found this induces cytolytic activity of CD4 T cells against tumor cells in vitro [283].

Conversely, cetuximab treatment has been linked to suppression of ADCC by increasing activity of regulatory T cells (Tregs), expressing CTLA-4, CD39, and TGFβ. Levels of Tregs correlated with poor clinical outcome in patient cohorts treated with cetuximab. Inhibition of CTLA-4(+) Treg by the CTLA-4-targeting antibody ipilimumab restored NK cell-mediated ADCC, resulting in better response to cetuximab treatment in vitro [284]. Finally, programmed death 1 (PD-1)/programmed death ligand 1 (PD-L1) immune checkpoint inhibitors are emerging as potent therapeutic agents. PD-1/PD-L1 limit T lymphocyte activation and promote immune resistance in SCCHN [285, 286]. In a cohort of 134 SCCHN specimens, PD-L1 levels were elevated and notably higher in HPV+ samples. This elevation was also positively correlated with EGFR and JAK2 levels; moreover, EGFR was identified as a mediator for activation of PD-L1 in a JAK2- and/or STAT1-dependent manner. In vitro studies using coculturing of tumor and NK cells showed that JAK2 inhibition using BMS-911543 in combination with cetuximab resulted in prevention of PD-L1 upregulation and increase of immunogenicity in tumor cells [287]. The rapid growth of the PD-1/PD-L1 inhibitor armamentarium and the large number of agents moving to clinical trial suggest that this field will expand enormously in the next several years.

2.5 Mechanisms of Resistance to Anti-EGFR Therapies

Patients initially responsive to anti-EGFR therapy often develop resistance during the course of treatment [18]. A number of specific factors associated with resistance have been identified [288]. These include altered ubiquitination and trafficking (Sect. 2.5.1) [289–291], overexpression and amplification of ErbB2 (Sect. 2.5.2) [216], altered expression levels of VEGF (Sect. 2.5.3) [292], altered expression levels of STAT3 (Sect. 2.5.4) [293], *KRAS* mutations (Sect. 2.5.5) [294, 295], changes in the tumor microenvironment (Sect. 2.5.6) [296], epigenetic compensation (Sect. 2.5.7) [297], and several other factors (Sect. 2.5.8).

2.5.1 EGFR-Intrinsic Resistance

Altered EGFR ubiquitination represents a mechanism of acquired resistance to cetuximab [289–291]. In vitro resistance to cetuximab was established by exposing cells to subeffective doses of cetuximab [291] or by prolonged exposure to escalating doses [290]. Lu et al. found that the cetuximab-resistant colorectal cancer cell line DiFi5 (rendered resistant through prolonged exposure to cetuximab) had markedly lower levels of EGFR. However, DiFi5 cells had enhanced associations between EGFR and the E3 ubiquitin ligase Cbl, as well as increased levels of ubiquitinated EGFR. DiFi5 also had significantly higher levels of active, Y16-phosphorylated Src, both at baseline and post-EGF stimulation, with inhibition of Src with the nonselective kinase inhibitor PP2 reversing cetuximab resistance. In addition, DiFi5 cells responded to EGF stimulation with more robust phosphorylation of EGFR at Y845 and strong phosphorylation of AKT and other extracellular EGFR signal-regulated kinases. These observations suggest that colorectal cancer cells may develop resistance to cetuximab by reducing EGFR levels via increased ubiquitination and degradation and via increased Src kinase-mediated cell signaling to bypass dependency on EGFR for cell growth and survival [291].

On the other hand, Wheeler et al. reported increased EGFR expression levels associated with deregulation of EGFR internalization and degradation in several resistant clones of NSCLC cell lines [289]. Loss of c-Cbl association with EGFR was reported to significantly lessen EGFR ubiquitination after EGF stimulation in the cetuximab-resistant cells compared to the nonresistant parent cells. These findings suggest that acquired resistance to cetuximab is accompanied by deregulation of EGFR internalization/degradation and subsequent EGFR-dependent activation of ErbB3 [289]. Further supporting the role of decreased EGFR ubiquitination in treatment resistance, Ahsan et al. found that cisplatin-resistant head and neck cancer cell lines undergo minimal EGFR phosphorylation at the Y1045 site and minimal ubiquitination [298].

Genetic variance in patient populations can affect response to EGFR inhibitors. For example, in >40% of patients with SCCHN, a single nucleotide variant polymorphism of *EGFR* (*EGFR R521K*) is present [299, 300]. This polymorphism has been linked to primary resistance to cetuximab in SCCHN in vitro and in vivo models and suggested as a potential marker for response to therapy. Cetuximab has a lower affinity to EGFR bearing a lysine residue at aa 521, affecting some patient populations. However, a next-generation EGFR antibody, GT-MAB 5.2-GEX with an Fc optimized by glycosylation [301, 302] to bind with higher affinity to the FcγRIIIa on immune effector cells and promote enhanced ADCC activity, targets EGFRK521 more efficiently [303].

2.5.2 Elevated Expression of ErbB Family Members

Ritter et al. demonstrated elevated levels of phosphorylated EGFR, EGFR/ErbB2 heterodimers, TGFα, hairpin-binding EGF, and heregulin RNA in trastuzumab-resistant human breast cancer cells. These findings suggest that enhanced EGFR-mediated activation of ErbB2 may be a potential mechanism of acquired resistance to trastuzumab [304]. A study by Yonesaka et al. (2011) identified a new mechanism of de novo and acquired resistance to cetuximab via increased signaling through ErbB2. Yonesaka et al. have shown that amplification of ErbB2 or upregulation of heregulin (ErbB3/ErbB4 ligand) is present in cetuximab-resistant colorectal cancer patients. This study suggests that ErbB2 inhibitors, in combination with cetuximab, represent a rational therapeutic strategy that should be assessed in patients with cetuximab-resistant SCCHN [212]. The same was demonstrated for ErbB3. In vitro experiments with SCCHN cell lines showed that treatment with cetuximab caused HER3 activation and HER2/HER3 dimerization. However, combined treatment with cetuximab and MM-121/seribantumab significantly decreased cell growth and downregulated the PI3K/AKT and ERK pathways, in comparison with each antibody alone. A similar effect was found in cetuximab-resistant xenografts and PDX models [305]. It is possible that such synergies may be achievable using a single therapeutic agent. A recent study in non-small cell lung cancer, using the novel Pan-HER inhibitor from Symphogen A/S (Ballerup, Denmark), which is an antibody mixture targeting EGFR, HER2, and HER3, effectively overcame resistance to cetuximab [214].

2.5.3 VEGF Expression

Enhanced angiogenesis is a fundamental step in the transition of tumors from a dormant state to a malignant one and correlates with tumor progression and metastasis [306]. Angiogenesis is elevated in various human tumors, including SCCHN, and VEGF has been demonstrated to be a major angiogenic factor [307]. Preclinical

and early clinical data imply a central role of angiogenesis in SCCHN: up to 90% of SCCHNs express vascular endothelial growth factor (VEGF) and the respective receptors (VEGFRs) [308].

Multiple studies support the prognostic implications of angiogenic markers in SCCHN and functional connections between angiogenic and EGFR signaling [309]. One recent study has identified angiopoietin-like 4 (ANGPTL4) as a regulator of EGF-induced cancer metastasis. The authors suggested that expression and autocrine production of ANGPTL4 mediated by EGF promotes SCCHN metastasis through the expression of metalloprotease-1 (MMP-1) [310]. Another study used immunofluorescence and single-cell segmentation to analyze expression and localization of EGFR in relation to the endogenous hypoxia marker CA IX and the intratumoral diffusion distance of EGFR+ cells from microvessels. Analysis was done using 58 human SCCHNs and a set of normal versus cancer-adjacent tissues [311]. This study found that the resistance to cetuximab was associated with downregulation of membrane EGFR expression in the hypoxic tissue [311]. Bossi et al. analyzed a group of recurrent-metastatic SCCHN patients pretreated with cetuximab and platinum chemotherapy, using whole transcriptome screening to find potential unique signatures of resistance. They found a connection between prolonged PFS and enriched expression of genes associated with active EGFR pathway signaling and hypoxic differentiation. In contrast, short PFS was associated with elevated RAS (but not EGFR) signaling, implying compensatory activation of downstream signaling [312].

EGFR activation and the overexpression of the three major ErbB-associated ligands trigger upregulation of multiple VEGF members and may induce resistance to anti-EGFR agents in vitro [292]. Riedel et al. showed that an EGFR antisense oligonucleotide treatment resulted in a significant reduction of VEGF protein expression, and addition of conditioned medium from EGFR antisense-treated tumor cells resulted in decreased endothelial cell migration [313]. The combination of bevacizumab (a humanized monoclonal IgG1 antibody targeting VEGF) with erlotinib was well-tolerated and had a response rate of 15% [314], which, in a cross-trial comparison, was higher than the response rate for erlotinib alone (5%) [315] or the VEGFR inhibitors SU5416 alone (5%) [316] or sorafenib (an inhibitor of VEGFR, PDGFR, Raf kinase, and others) alone (3–4%) [317]. A phase II clinical trial of sorafenib in combination with cetuximab demonstrated no clinical benefit in a group of patients with recurrent and/or metastatic (R/M) SCCHN [318]. Argiris et al. demonstrated that the combination of bevacizumab and cetuximab enhanced growth inhibition both in vivo and in vitro in preclinical models and resulted in a SCCHN disease control rate of 46% [319]. However, a phase II trial showed that radiotherapy and cetuximab with the addition of bevacizumab led to no improvement in treatment efficacy compared with radiation/cetuximab alone, showing no clinical benefits of dual targeting of VEGF and EGFR for a group of previously untreated stage III–IVB SCCHN patients [320]. Chemotherapy with or without bevacizumab is being investigated in a phase III trial with patients with recurrent or metastatic head and neck cancer (NCT00588770).

A novel fully humanized dual-targeting IgG (DT-IgG) antibody that simultaneously targets VEGF and EGFR has been designed to optimize tumor targeting and

maximize potential clinical benefits [321]. Hurwitz et al. tested DT-IgG on SCCHN, lung adenocarcinoma, and colon cancer xenograft models and discovered that DT-IgG had a lower in vivo IC50 than bevacizumab (VEGF-targeting antibody) and cetuximab; however, a higher dose of DT-IgG was needed to produce efficacy similar to that observed with combined bevacizumab and cetuximab treatment [321].

Zhang et al. showed in SCCHN in vitro studies that DT-IgG neutralizes VEGF as effectively as bevacizumab and inhibits EGFR activation and cell proliferation as effectively as cetuximab [322]. One obvious benefit of DT-IgG therapy would be avoidance of dosing complications associated with drug combinations [321, 322]. Lecaros et al., using human SCCHN xenograft models, have found that delivery of siRNA against VEGF-A using lipid-calcium-phosphate nanoparticles (LCP NPs) in combination with photodynamic therapy promotes apoptosis and controls tumor growth [323]; this may be useful in conjunction with EGFR-targeting therapies.

2.5.4 STAT3 Expression

Sen et al. found that increased STAT3 may contribute to cetuximab resistance in SCCHN [293]. STAT3 inhibition in cetuximab-resistant SCCHN cells using a STAT3 decoy oligonucleotide to inhibit STAT3-mediated transcription reduced cellular viability and the expression of STAT3 target genes. STAT3 decoy treatment also successfully decreased tumor growth in vivo [293]. In SCCHN cells, activation of the JAK2/STAT3 pathway was associated with EMT and metastasis through induction of the chemokine CCL19 and its receptor CCR7, which was previously found to play an important role in chemotaxis and migration of immune cells, such as leukocytes. In a panel of 78 human SCCHN specimens, phosphorylation of CCR7 and STAT3 positively correlated with lymph node metastasis [324].

2.5.5 KRAS *and* PI3K *Mutation*

KRAS mutations are fairly rare in SCCHN compared to other types of cancer [325]. Mutational activation of KRAS only occurred in 2.6% of 115 clinical specimens of SCCHN, although copy number amplification of *KRAS* was found in 10 samples (8.7%) in the same study [326]. Chau et al., using next-generation sequencing (NGS) in a group of 213 SCCHN patients, demonstrated that oncogenic *RAS* mutations are associated with poorer PFS. In another study, *KRAS* mutations were found in 4 out of 29 patients with SCCHN, and the presence of the *G12 V KRAS* mutation was associated with an absence of response to cetuximab and radiotherapy [327]. Monitoring of *RAS* mutations as well as mutations in the cetuximab-interacting ectodomain of the EGFR may be a good predictive marker for cetuximab

resistance [328, 329]. *KRAS* mutation has been associated with HPV status. In a cohort of 179 SCCHN, *KRAS* mutations occurred more frequently in HPV(+) tumors (6%) versus 1% for HPV(-) tumors [330]. Finally, as noted above, *PIK3CA* is one of the most frequently amplified or mutated oncogenes in SCCHN tumors [67, 119, 121], with mutation associated with activation of the AKT signaling pathway [331]. Additionally, phosphatase and tensin homolog (PTEN) acts as a tumor suppressor and an important negative regulator of the PI3K/AKT pathway. Low expression of PTEN (44.4% with low vs. 71.7% with high) and pAKT (75.2% with low vs. 52.5% with high) were shown to be important for prolonged 2-year overall survival in a group of 49 patients with SCCHN previously treated with cetuximab-based induction chemotherapy (a combination of cetuximab, docetaxel, and cisplatin) [332]. Burtness et al. demonstrated that loss of PTEN (PTEN null) was observed in 23 of 67 (34%) SCCHN patient samples analyzed by AQUA and that treatment of these patients with cetuximab or placebo leads to better PFS in comparison with patients with tumors expressing PTEN (4.2 months for cetuximab vs. 2.9 months for placebo for patients expressing PTEN and 4.6 months for cetuximab vs. 3.5 months for placebo for PTEN null) [333].

2.5.6 Microenvironment

A growing body of evidence suggests that components of the tumor microenvironment may also contribute to tumorigenesis in cancers of epithelial origin and may modulate the treatment sensitivity of tumor cells [334]. Johansson et al. reported that cancer-associated fibroblasts (CAFs) offer protection from cetuximab treatment and negate cetuximab-induced growth inhibition [296]. They further described that SCCHN cell lines cocultured with CAFs from patients with SCCHN result in elevated expression of matrix metalloproteinase-1 (MMP-1) in both the tumor cells and the CAFs. MMP inhibitors can partly abolish CAF-induced resistance; however, siRNA knockdown of MMP-1 in CAFs did not abolish resistance, suggesting that other MMP family members may be involved (Johansson et al. 2012). The mechanism of MMP-associated cetuximab resistance is not clear, and further investigation is warranted. Quantitative RT-PCR analysis of 31 tumor-adjacent tissues, 94 SCCHN tumors, and 10 tonsillectomy noncancerous specimens has revealed that expression of metalloproteins (MT) MT1A and MT2A is significantly higher in the tumors and correlates with a higher tumor grade. Expression of MT was also observed in tonsillectomy samples, gradually increasing in adjacent tissues and tumors, possibly in response to the oxidative stress. The authors suggested that MT accumulation in adjacent tissue occurs as a response to the tumor cells [335].

2.5.7 Epigenetic Changes

Emerging evidence has indicated connections between epigenetic changes, such as DNA methylation at CpG islands, and development of resistance to multiple cancer therapeutics [336–339]. Ogawa et al. tested a panel of 56 genes (including death-associated protein kinase (*DAPK)*, *MGMT*, and *SRBC*, commonly known to be regulated through promoter methylation, using array-based methylation analysis of two parental NSCLC and SCCHN cell lines and progeny rendered resistant to either erlotinib or cetuximab. The study found that DAPK was hypermethylated in NSCLC and SCCHN drug-resistant cells. Subsequent demethylation of DAPK in the resistant NSCLC cells restored sensitivity to both erlotinib and cetuximab. siRNA-mediated knockdown of DAPK validated the array-based findings by inducing erlotinib and cetuximab resistance in cells normally sensitive to either agent [297].

2.5.8 Other Factors

Transforming growth factor beta (TGFβ) has recently been shown to be a key molecular determinant of de novo and acquired resistance of cancers to EGFR-targeted antibodies [340]. Bedi et al. found that treatment of mice bearing xenografts of human SCCHN cells with cetuximab resulted in emergence of resistant tumor cells that expressed relatively higher levels of TGFβ compared to the control group. Also, treatment with cetuximab alone induced an apparent natural selection of TGFβ-overexpressing tumor cells in nonregressing tumors. Combinatorial treatment with cetuximab and a TGFβ-blocking antibody prevented the emergence of resistant tumor cells and induced complete tumor regression [340].

Coexpression of elevated levels of Aurora-A and EGFR was an adverse prognostic factor with poor disease-free and overall survival in a cohort of 180 patients [341]. In vitro studies showed that simultaneous targeting of Aurora kinase and EGFR using cetuximab and a pan-Aurora kinase inhibitor (R763) was more effective than mono-EGFR or mono-Aurora kinase inhibition. Interestingly, growth inhibitory effects were noticeable with the addition of R763 to cell lines with no or very moderate response to mono-EGFR-targeted treatment and/or with very low EGFR expression [341]. Independent studies have shown efficacy of combination of a specific Aurora-A inhibitor with erlotinib and cetuximab in an EGFR-dependent cancer cell line [342]. These findings suggest that Aurora kinase inhibitors may help overcome cetuximab resistance in the treatment of SCCHN; however, more work is needed.

Recent studies have revealed a connection between a fibroblast growth factor receptor 3-transforming acidic coiled-coil-containing protein 3 (FGFR3-TACC3) fusion protein and drug resistance. This fusion gene has been detected in nasopharyngeal carcinoma, SCCHN, esophageal squamous cell carcinoma, and lung cancer. In vitro studies have identified that FGFR3-TACC3 fusion gene promotes survival of cancer cells [343]. In a murine SCCHN xenograft model, simultaneous targeting

of both EGFR and ERBB3 leads to decreased tumor progression. However, it results in the outgrowth of resistant cells. Daly et al. revealed that in these tumors levels of FGFR3-TACC3 fusion protein are significantly elevated. Further investigation demonstrated that FGFR3-TACC3 overexpression promotes cancer cell resistance through the blockade of EGFR/RAS/ERK signaling, but not through ERBB3/PI3K/AKT signaling [344].

2.6 Toxicity and Tolerance

EGFR is widely expressed at the basal level of the epidermis [293, 294], and EGFR-directed antibodies have predictable dermatologic side effects including follicular eruption, scaling, paronychia, and skin fissures. Although a significant difference in high-grade in field skin toxicity was not reported by Bonner et al. [175], subsequent studies have demonstrated increased high-grade radiation dermatitis and mucositis [176]. Initial management of skin toxicity is with topical steroids; antibiotics may be indicated in the case of superinfection, and systemic steroids are reserved for desquamation or very high proportion of body surface involved [345]. Skin toxicity is associated with benefit from cetuximab in both SCCHN and colon cancer [193, 295]. Interestingly, there is some evidence that intrinsic germline genetic variation of *EGFR* (rs2227983), *KRAS* (rs61764370), and *FCGR2A* (rs180127) can be used as predictive markers of reduced skin toxicity in SCCHN patients treated with a cetuximab-based therapy [346]. Allergic and anaphylactoid reactions can be observed in patients treated with cetuximab and, less often, with panitumumab; however, both of these monoclonal antibodies have fewer nonspecific and hematopoietic side effects compared to other chemotherapeutics [347, 348]. Electrolyte abnormities, specifically hypomagnesemia, are also commonly observed and should be monitored during treatment with EGFRIs [195].

2.7 Conclusions and New Frontiers in Drug Discovery

As study of EGFR and the ErbB family advances, several themes emerge for future investigation.

First, major, ongoing investments in personalized medicine, involving in part studies of exceptional responders for targeted therapy, and growth of large databases integrating sequence and clinical data, will help provide a panel of predictive response biomarkers. However, based on data summarized above, interpreting these data will be a challenge. It is likely that there will be multiple potential mechanisms for resistance, each operative in small subsets of patients: further, given that targeted therapies are typically administered in combinations, and many combinations are under evaluation, it will be challenging to develop statistically robust datasets to predict response to drug combinations. Systems biology may be able to help guide

the integration of this information [349]. In complementary work, high-throughput screening may identify new targets relevant to specific subtypes of SCCHN. In another example, recent studies applying whole exome sequencing to nasopharyngeal cancers identified specific mutational signatures in this disease subclass, some of which are potentially druggable and may provide patient-tailored options [350] that can augment classic EGFR-ErbB-targeted therapeutics.

Second, existing EGFR-/ErbB-targeting agents may find new uses in the fields of SCCHN prevention, while efforts continue to develop new therapeutic strategies for EGFR and ErbB. In some intriguing recent work, and echoing the fact that some studies have found elevated EGFR in noncancerous premalignant tissue [11, 12, 351], cetuximab treatment of patients with high-risk premalignancy of the upper aerodigestive tract was demonstrated as a potentially effective treatment of moderate to severe dysplasia in a subset of patients [352]. However, a conceptually related study using erlotinib was not effective [351], and more work is required. In terms of developing new approaches to targeting EGFR, a number of studies support the idea that EGFR forms higher-order oligomers, such as tetramers, during signaling [353–355]. Ramirez et al. used a virtual docking model of extracellular tetrameric EGFR configuration coupled with functional screening to identify compounds that affect internalization of adaptor responsive to EGFR activation Grb2. This work indicates it may be possible to develop new classes of EGFR-targeting agents, based on targeting epitopes formed by functional, higher-order oligomers [356].

Third, the rapid rise of immunotherapies, with results from major trials expected in the next several years, is likely to transform the use of EGFR-/ErbB-targeting therapies. As effective immunotherapies have only recently been developed, much effort will be needed to understand how these agents interact with EGFR- or ErbB-targeting agents and cytotoxic therapies, develop appropriate dosing regimens that maximize effectiveness and minimize toxicities, establish genomic signatures and clinical profiles that are best suited for their use, and many other considerations. It is to be hoped that successful integration of these new approaches with EGFR-targeting agents will ultimately result in better patient outcomes.

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