Current Cancer Research

Barbara Burtness · Erica A. Golemis
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Molecular Determinants of Head and Neck Cancer

Second Edition

Humana Press

Current Cancer Research

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ISSN 2199-2584 ISSN 2199-2592 (electronic)
Current Cancer Research
ISBN 978-3-319-78761-9 ISBN 978-3-319-78762-6 (eBook)
https://doi.org/10.1007/978-3-319-78762-6

Library of Congress Control Number: 2018946649

This Humana Press imprint is published by the registered company Springer International Publishing AG part of Springer Nature.
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

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Chapter 1
Overview: The Pathobiology of Head and Neck Cancer

Barbara Burtness and Erica A. Golemis

Squamous cell cancers arising in the head and neck, from the nasopharynx to the subglottic larynx, are frequently devastating cancers that afflict patients around the world. Early stage cancers are readily cured with surgery or radiation. In contrast, locally advanced cancers require morbid multimodality therapy and nonetheless have high recurrence rates, while metastatic disease has not been curable with cytotoxic chemotherapy. The emergence of more treatment-responsive human papillomavirus (HPV)-driven cancers and the advent of immune checkpoint blockade mean that the outlook for patients with head and neck cancer has improved dramatically since the first edition of this book was published in 2014. Our understanding of the biology of this cancer has deepened considerably in the past 4 years, yet undruggable targets due to the predominance of tumor suppressor gene mutation and other noncatalytic abnormalities continue to present barriers to molecular and personalized therapy and to cure in HPV-negative disease.

The second edition of *Molecular Determinants of Head and Neck Cancer* addresses this difficult disease with a focus on the molecular processes in the carcinogenesis and progression of these cancers which will inform the search for therapeutic targets to enable the prevention and improve the cure of head and neck cancer. With the current volume, we introduce the etiology and subclasses of head and neck squamous cell carcinomas (HNSCC), in the context of how these differences affect prognosis. Second, we summarize the current state of understanding of the genetic, epigenetic, protein expression, and immune environmental changes associated with SCCHN. Thirdly, we situate novel targets in the context of these

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B. Burtness, E. A. Golemis (eds.), *Molecular Determinants of Head and Neck Cancer*, Current Cancer Research, https://doi.org/10.1007/978-3-319-78762-6_1

insights into SCCHN, seeking to provide a template for development of novel treatment strategies.

We begin by introducing the receptor tyrosine kinases (RTKs) and signaling pathways which are central to the biology of HNSCC. Chapter 2 describes the central role of ERBB/HER family proteins in the biology of head and neck cancer and reviews the data regarding inhibition of HER family signaling, given that EGFR remains the sole validated molecular target in HNSCC [1, 2]. Upregulation of RTKs with partially redundant function may provide resistance to cetuximab and more recently developed EGFR-targeting therapies. In particular, abnormal expression and activation of RTKs such as c-MET [3] have emerged as relevant to the pathology of HNSCC and may prove to be an important therapeutic target in this disease (Chap. 3).

Tumor invasion depends in part on epithelial-mesenchymal transition (EMT), which occurs in response to activation of transforming growth factor β (TGFβ) [4], a transmembrane serine-threonine kinase, and its canonical and noncanonical effectors. This is reviewed in Chap. 4. A common feature of RTKs is their activation of downstream effector pathways that support tumor growth, survival, and resistance to therapy. In the case of SCCHN, some of the most important of the effectors are themselves mutated or otherwise constitutively activated. Chapter 5 details mutational and indirect activation of the PTEN-PI3K-AKT-mTOR [5] signaling axis in HNSCC with efforts to target this pathway; while Chap. 6 discusses the role of constitutive JAK/STAT signaling [6] observed in a subset of HNSCC, with the challenges in targeting these noncatalytic signaling proteins. Conversely, Chap. 7 addresses the multiple defects in cell cycle regulation that occur in HNSCC and offer another potential source of targetable vulnerability.

One of the challenges in molecularly directed therapy of HNSCC has been the predominance of tumor suppressor mutations in this disease. Notch signaling is implicated in multiple cellular functions associated both with cancer and with tumor suppression. Notch mutation is a frequent event in HNSCC [7]; however, interestingly, the majority of these mutations are inactivating, indicating that the tumor suppressive function dominates in this epithelial tissue; this is reviewed in Chap. 8. The tumor suppressor *TP53* has long been understood to be the most commonly mutated gene in HPV-negative HNSCC and a major contributor to therapeutic resistance [8, 9]. The biology of this tumor suppressor, as well as new strategies to exploit loss of p53 function with synthetic lethal strategies, are described in Chap. 9.

The entire field of cancer biology is being transformed by the application of powerful new technologies that are elucidating the genome and epigenome. Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) proteins are gene editing enzymes which are upregulated in response to viral infection as well as to replication stress and result in mutations and characteristic patterns of mutation. APOBEC family members are active in both HPV-related and HPV-negative HNSCC [10, 11], and both the protein and the mutational burden it elicits have therapeutic implications, discussed in Chap. 10. The theme of insights into the head and neck cancer genome, and the nature of intrinsic HNSCC subsets,

is continued in Chap. 11, and the epigenome, with the impact of gene expression, methylation, and histone structure, is considered in Chap. 12.

Since the first edition of this book, the role of inflammation as a factor conditioning growth of tumor cells and the growth-promoting aspects of the tumor microenvironment has taken on much greater prominence. Chapter 13 addresses the process of inflammation as a contributor to tumor aggressiveness, based on activities on tumor cells and immune cells in the tumor microenvironment, and addresses the potential of the inflammatory process as a source of new targets for therapy. In the clinic, the advent of agents to block the immune checkpoint has led to new therapies and a flood of clinical trials. The complex immune microenvironment of HNSCC and the multiplicity of investigational immunotherapy approaches to reverse immune tolerance [12] are reviewed in Chap. 14. Neovascularization and hypoxia have been associated with treatment resistance in HNSCC [13]. Hypoxia-inducible factor is reviewed in Chap. 15, and vascular-endothelial growth factor (VEGF) and the agents which target this angiogenic factor and regulate tumor vascularization [14] are reviewed in Chap. 16.

The theme of the tumor environment and epithelial-mesenchymal transition is continued with chapters discussing integrin-mediated signaling, which mediates interactions between the tumor and the extracellular matrix (ECM), and the stem cell niche. Focal adhesion kinase (FAK) is a key mediator of integrin signaling, mediating tumor-ECM communications in HNC in a manner that affects treatment response [15]: biology of FAK and integrin and the clinical prospects of their inhibitors are the subject of Chap. 17. The Wnt/β-catenin signaling pathway [16] provides an independent input into cell differentiation status, affecting EMT, cancer stem cells, and therapeutic resistance. A growing body of evidence supports the common deregulation of expression of Wnt signaling proteins in HNSCC [16], with early efforts to evaluate therapeutic agents targeting some signaling intermediates. Wnt signaling is presented in Chap. 18 and hyaluronan-mediated activation of head and neck cancer stem cells in Chap. 19.

Historically, habitual exposures such as tobacco, alcohol, and mate have contributed to onset of SCCHN. However, a rising proportion of oropharynx cancers arise from transforming HPV infection [17]. Chapter 20 presents the epidemiology of the various types of HPV that contribute to SCCHN pathogenesis and assesses the potential for targeting viral oncoproteins. For HPV-associated SCCHNs, it will be necessary to identify biomarkers to distinguish between patients with near certainty of cure, and those – perhaps most commonly smokers – with HPV-associated cancer but a higher risk of recurrence [18]. Reduced treatment intensity and concomitant reduction of treatment-related morbidity may be achievable for the former; diverse approaches to treatment-deintensification are the focus of Chap. 21.

The 4 years since the first edition of this book have been marked by increased confidence that treatment for some subsets of HPV-driven HNSCC can be scaled back, by a revolution in our ability to manipulate the immune response to cancer, and by tantalizing clues that the PI3K and angiogenic pathways may also constitute valuable targets in HNSCC. However, HPV-negative HNSCC has not been readily amenable to targeted therapy, and even immunotherapy has had more modest effects

in this cancer than in other solid tumors. Patients with HNSCC need treatments that exploit our advancing understanding of the biology of this malignancy. As the chapters collected here make clear, the advances in understanding this cancer bring us progressively closer to improved therapies for many subsets of HNSCC. Going forward, rapid translation of these findings to clinical trials will be essential to extend these insights to the cure of human head and neck cancer.

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Chapter 2
Targeting the ErbB Family in Head and Neck Cancer

Anna Kiseleva, Tim N. Beck, Ilya G. Serebriiskii, Hanqing Liu, Barbara Burtness, and Erica A. Golemis

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%

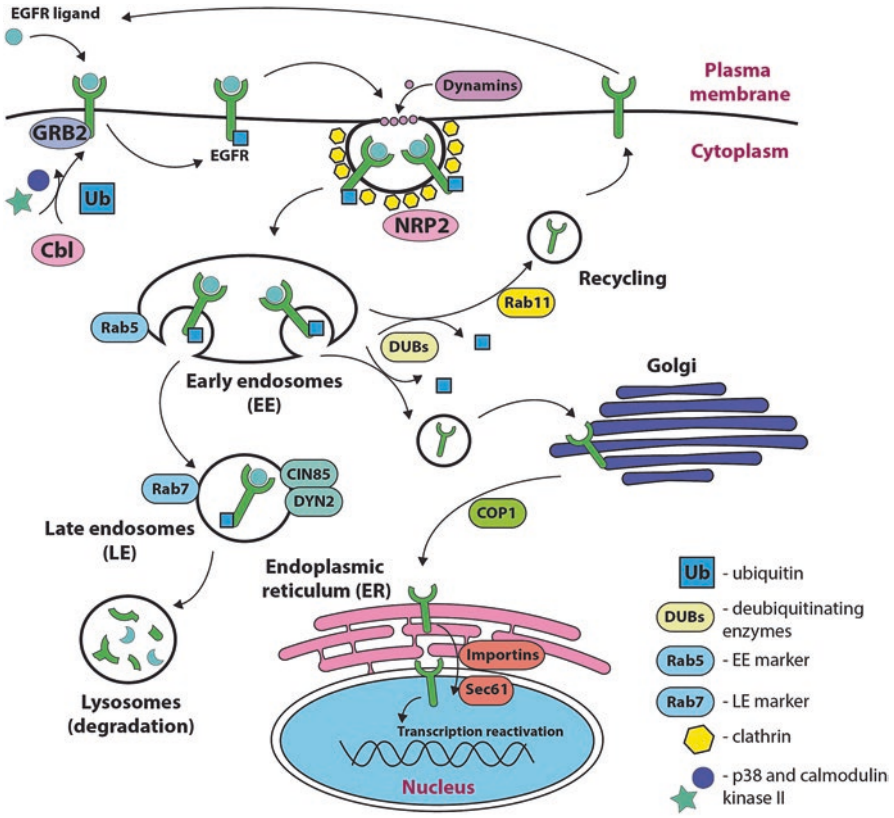


Fig. 2.1 Regulation of internalization and degradation of ErbB proteins. Here we described clathrin-mediated endocytosis of EGFR with further transportation to late endosomes and degradation, recycling, or migration to the nucleus

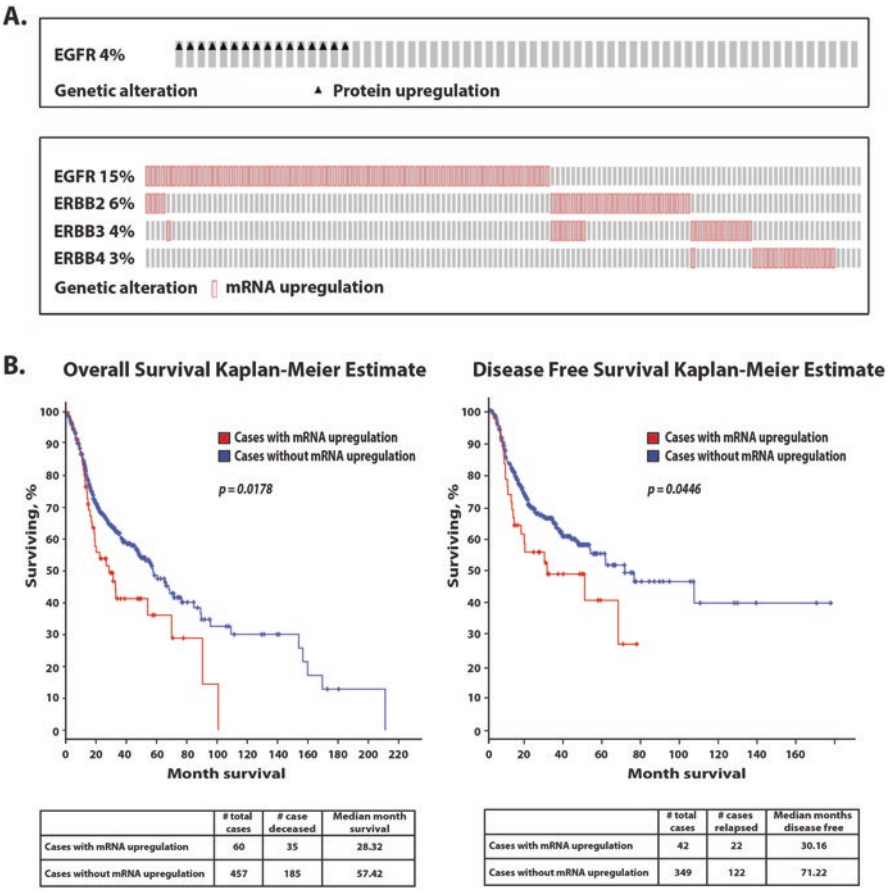
in a group of 91 patients with SCCHN [57]. In another study, EGFR protein levels in 140 primary laryngeal squamous cell carcinomas were determined using a radioligand receptor assay. The authors established that a cutoff value of 20 fmol mg^{-1} is an effective prognostic marker, and based on this classification, 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

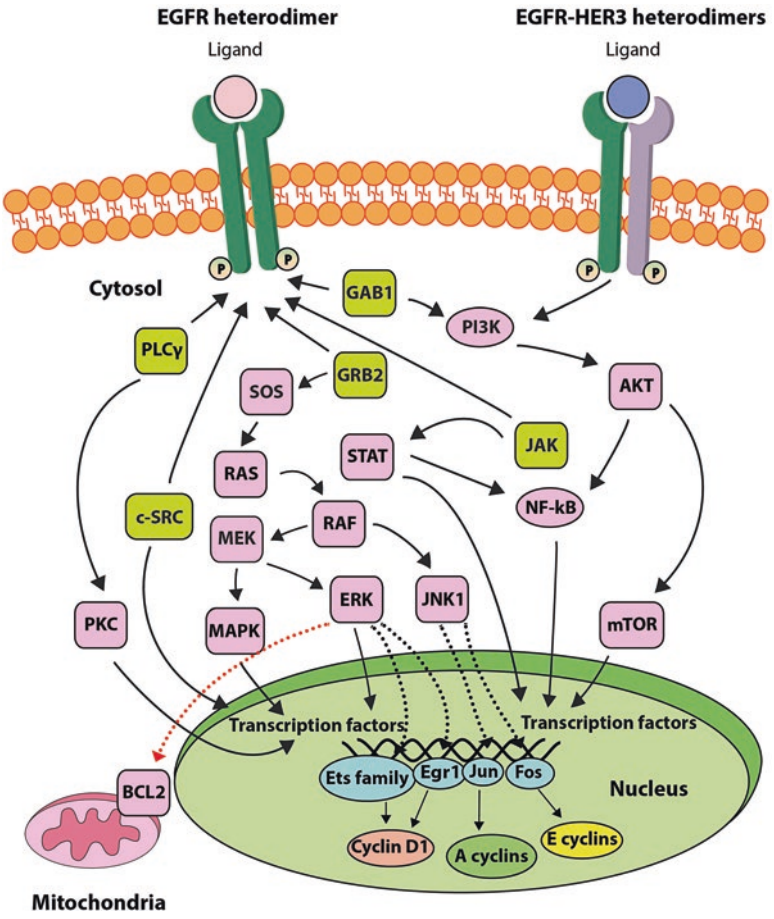


Fig. 2.3 Signaling pathways downstream of EGFR and other ErbB proteins that have been linked to tumorigenesis of SCCHN and/or resistance to ErbB-targeting inhibitors. *Green* boxes indicate targets which bind directly to the EGFR phosphorylation sites. See text for details

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