Overcoming Resistance to EGFR inhibitor in Head and Neck Squamous Cell Carcinoma (HNSCC)

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1 Introduction

HNSCC (head and neck squamous cell carcinoma) is the sixth most common neoplasm worldwide, with approximately 600,000 patients newly diagnosed each year (Cripps et al., 2010). Over the past 30 years, patients with recurrent and/or metastatic HNSCC have had a poor prognosis (Forastiere et al., 2001; Khuri et al., 2000). More than 50% of newly diagnosed patients do not achieve complete remission, and approximately 10% relapse with metastasis to distant organs (van Houten et al., 2000). Therefore, research focused on gaining a better understanding of this disease and on the development of novel treatment strategies is required.

Epidermal growth factor receptor (EGFR), a ubiquitously expressed transmembrane glycoprotein belonging to the ErbB/HER family of receptor tyrosine kinases (TK), is composed of an extracellular ligand-binding domain, a hydrophobic transmembrane segment and an intracellular TK domain. Upon ligand binding to EGFR, the latter undergoes a conformational change that promotes homo- or heterodimerization with other members of the ErbB/HER family of receptors, followed by autophosphorylation and activation of the TK domain (Ciardiello & Tortora, 2003). Activation of EGFR leads to activation of intracellular signaling pathways that regulate cell proliferation, invasion, angiogenesis and metastasis.

EGFR is expressed at high levels in the majority of epithelial malignancies including HNSCC (Saranath et al., 1992). Elevated expression of EGFR in HNSCC correlates with poor prognosis, and EGFR has been a target of anticancer treatments due to its critical roles in cell survival and proliferation (Burtness, 2005). Among the tyrosine kinase inhibitors targeting EGFR that have been approved by the U.S. FDA are gefitinib, erlotinib and lapatinib (Carter et al., 2009). These molecules are reversible competitors, competing with adenosine triphosphate (ATP) for the tyrosine kinase binding domain of EGFR. Inhibition of receptor activation inhibits downstream signaling pathways, resulting in decreased cell proliferation and survival. The pathways mediating EGFR downstream effects have been well studied and three major signaling pathways have been identified, with the first involving the RAS-RAF-MAPK pathway. Upon ligation, ErbB receptors are activated. The activated receptor kinase phosphorylates tyrosine residues at the C-terminal end of ErbB receptors, allowing the binding of proteins containing the Src Homology 2 (SH2) domain, including intracellular docking and adaptor proteins, such as Grb2 and Shc. Upon binding to ErbB receptors, these proteins associate with other proteins, leading to the activation of serine-threonine kinases that phosphorylate serine and threonine residues on other protein kinases and/or transcription factors. This kinase cascade leads to the amplification of a network of signaling pathways, resulting in changes in protein function and activation of gene transcription. Mitogen activated protein kinases (MAPKs) are a superfamily of protein serine-threonine kinases, including Erk1/2. ErbB receptors activate Erks by binding to the adaptor protein Grb2, recruiting son of sevenless (SOS) protein to the receptor. SOS is a guanyl nucleotide-release protein (GNRP), which upon recruitment to the plasma membrane by activated cell surface receptors, causes the small G protein RAS to release GDP and bind GTP, resulting in Ras activation. Activation of Ras leads to the activation of the MKKK Raf-1, which, in turn, phosphorylates and activates the MKK Mek1/2. Activated Mek1/2 then phosphorylates and activates Erk1/2. This cascade results in the phosphorylation of a variety of substrates including 90 kDa ribosomal S6 protein kinase (Rsk), Msk1, cytosolic phospholipase A2, and the transcription factors c-Myc, NF-IL6, Tal-1, Ets-2, and Elk. This results in enhanced gene transcription and increased cell proliferation.

The second major signaling pathway mediating EGFR downstream effects is the PI3K/AKT pathway, which activates the major cellular survival and anti-apoptosis signals via activating nuclear transcription factors such as nuclear factor-kappa B (NF-kB), whereas the third major signaling pathway is
the JAK/STAT pathway, which has also been implicated in activating the transcription of genes associated with cell survival.

2 Rare EGFR Mutations in HNSCC

Somatic mutations in the TK domain of the EGFR gene, including in-frame deletions in exon 19 and the point mutations L858, G719X and L861Q, have been associated with increased sensitivity to EGFR TK inhibitors (TKIs) and are present in 10–30% of patients with non-small cell lung carcinoma (NSCLC), depending on ethnic origin. These mutant EGFRs selectively activate the signal transduction and activator of transcription (STAT) and Akt signaling pathways, which promote cell survival. However, they have no effect on extracellular signal-regulated kinase (ERK) signaling, which induces cell proliferation. Furthermore, mutant EGFRs selectively transduce survival signals, and inhibition of these signals may contribute to the efficacy of TKIs used to treat NSCLC (Sordella et al., 2004). However, molecular analysis of HNSCC tumor samples has not revealed the same spectrum of mutations (Loeffler-Ragg et al., 2006; Ozawa et al., 2009; Taguchi et al., 2008).

One important resistance mutation in EGFR is the T790M missense mutation in the kinase domain, which may contribute to TKI resistance in NSCLCs possessing the L858R point mutation (Wong, 2008). Using the cycleave PCR method, however, we failed to detect the T790M mutation in 86 HNSCC tumor samples (Baba et al., 2012).

3 Resistance to EGFR TKIs

EGFR TKIs have had limited results in patients with HNSCC. For example, a phase II trial of gefitinib in patients with recurrent or metastatic HNSCC showed an overall response rate of 11% (Cohen et al., 2003). Similarly, a study of erlotinib in patients with recurrent and/or metastatic HNSCC showed a response rate of 4% (Soulieres et al., 2004). Four mechanisms have been proposed to explain tumor resistance to EGFR TKIs.

1. **Ras mutations** K-ras mutations may cause tumor insensitivity to EGFR TKIs. Activating K-ras mutations may activate the Ras/mitogen activated protein kinase (MAPK) pathway independent of EGFR, thus inducing resistance to EGFR TKIs (Eberhard et al., 2005). H-ras mutations are more common than K-ras mutations in HNSCC and may play an important role in tumor resistance to EGFR-targeted therapies (Anderson et al., 1994).

2. **Epithelial-mesenchymal transition (EMT)** EMT results in changes in cell morphology and motility and is indicated by increased expression of vimentin and claudins 4 and 7 and by decreased expression of E-cadherin. EMT has been associated with gefitinib resistance in HNSCC (Frederick et al., 2007).

3. **Upregulation of cyclin D1** Upregulation of cyclin D1 in HNSCC cell lines has been specifically associated with resistance to gefitinib. Upregulation of cyclin D1 results in the activation of cyclin D1-cyclin dependent kinase 4 (CDK4), which hyperphosphorylates retinoblastoma protein (pRb) (Kalish et al., 2004).
4. PI3K/Akt signaling as a dominant pathway

Increased expression of cortactin, a protein that increases the formation of actin networks critical to cell motility and receptor-mediated endocytosis, has been associated with gefitinib resistance and increased metastasis in HNSCC (Timpson et al., 2007).

Akt has been implicated in EMT by integrin-linked kinase (ILK). The PI3K/Akt pathway not only regulates the transcriptional activity of cyclin D1, but increases its accumulation by inactivating glycogen synthase kinase-3 (GSK3), an enzyme that targets cyclin D1 for proteasomal degradation. Cortactin is thought to promote cancer cell proliferation by activating Akt (Timpson et al., 2007), suggesting that factors related to resistance to EGFR TKIs are associated with the PI3K/Akt pathway.

4 PI3K/Akt Pathway

In this section, we will explain the activation of the PI3K/AKT pathway, its downstream effectors, and the rationale for targeting this pathway in HNSCC.

4.1 Activation of the PI3K/Akt Pathway

Signaling through the PI3K/Akt pathway can be initiated by several mechanisms. Once activated, this pathway can be propagated to various substrates, including mTOR, a master regulator of protein translation. The PI3K/Akt pathway is initially activated at the cell membrane, where the signal for activation is propagated through class IA PI3K. Activation of PI3K can occur through tyrosine kinase growth factor receptors such as EGFR and insulin-like growth factor-1 receptor (IGF-1R), cell adhesion molecules such as integrins, G-protein-coupled receptors (GPCRS) and oncogenes such as Ras. PI3K catalyzes the phosphorylation of the D3 position on phosphoinositides, generating the biologically active moieties phosphatidylinositol-3,4,5-triphosphate (PI(3,4,5)P3) and phosphatidylinositol-3,4-bisphosphate (PI(3,4)P2).

PI(3,4,5)P3 binds to the pleckstrin homology (PH) domains of 3’-phosphoinositide-dependent kinase 1 (PDK-1) and Akt, resulting in the translocation of these proteins to the cell membrane, where they are subsequently activated. The tumor suppressor phoshatase and tensin homolog deleted on chromosome ten (PTEN) antagonizes PI3 kinase by dephosphorylating PI (3,4,5)P3 and (PI(3,4)P2), thereby preventing the activation of Akt and PDK-1. Akt exists as three structurally similar isoforms, Akt1, Akt2 and Akt3, which are expressed in most tissues. Activation of Akt1 occurs through two crucial phosphorylation events. The first, catalyzed by PDK-1, occurs at T308 in the catalytic domain of Akt1. Full activation requires a subsequent phosphorylation at S473 in the hydrophobic motif of Akt1, a reaction mediated by several kinases, including PDK-1, ILK, Akt itself, DNA-dependent protein kinase and mTOR; phosphorylation of homologous residues in Akt2 and Akt3 occurs by the same mechanism. Phosphorylation of Akt at S473 is controlled by a recently described phosphatase, PH domain leucine-rich repeat protein phosphatase (PHLPP), which has two isoforms that preferentially decrease the activation of specific Akt isoforms (Brognard et al., 2007). Amplification of Akt1 has been described in human gastric adenocarcinomas, and amplification of Akt2 has been described in ovarian, breast and pancreatic carcinomas (Bellacosa et al., 1995; Cheng et al., 1996). Akt mutations are rare, but somatic mutations in the PH domain of Akt1 have been detected in small percentages of human breast, ovarian and colorectal cancers (Carpten et al., 2007).
4.2 Downstream Substrates of Activated Akt

Akt recognizes and phosphorylates the consensus sequence RXRXX (S/T) when it is surrounded by hydrophobic residues. Since this sequence is present in many proteins, Akt has many substrates, many of which control key cellular processes such as apoptosis, cell cycle progression, transcription and translation. For example, Akt phosphorylates proteins in the FoxO subfamily of forkhead family transcription factors, inhibiting the transcription of several pro-apoptotic genes, including Fas-L, IGF binding protein1 (IGFBP1) and Bim. In addition, Akt can directly regulate apoptosis by phosphorylating and inactivating pro-apoptotic proteins such as BAD, which controls the release of cytochrome c from mitochondria, and apoptosis signal-regulating kinase-1 (ASK1), a mitogen-activated protein kinase involved in stress- and cytokine-induced cell death. In contrast, Akt can phosphorylate IKK, which indirectly increases the activity of nuclear factor kappa B (NF-κB) and stimulates the transcription of pro-survival genes. Cell cycle progression can also be affected by Akt; inhibitory phosphorylation of the cyclin-dependent kinase inhibitors p21 and p27 and of GSK3β by Akt has been found to stimulate cell cycle progression by stabilizing cyclin D1 expression. Akt phosphorylation of a recently described, novel pro-survival Akt substrate, proline-rich Akt substrate of 40 kDa (PRAS40), has been shown to attenuate the ability of the latter to inhibit mTORC1 kinase activity (Vander Haar et al., 2007). PRAS40 may be a specific substrate of Akt3 (Madhunapantula et al., 2007). These findings therefore indicate that Akt inhibition may have pleiotropic effects on cancer cells that contribute to an anti-tumor response. The most-studied downstream substrate of Akt is the serine/threonine kinase mammalian target of rapamycin (mTOR). Akt can directly phosphorylate and activate mTOR, as well as indirectly activating it by phosphorylating and inactivating tuberous sclerosis complex 2 (TSC2), also called tuberin, which normally inhibits mTOR through the GTP binding protein Ras homolog enriched in brain (Rheb) (Inoki et al., 2003). When TSC2 is inactivated by phosphorylation, the GTPase Rheb is maintained in its GTP-bound state, allowing increased activation of mTOR (Inoki et al., 2005). mTOR exists in two complexes: the TORC1 complex, in which mTOR is bound to Raptor; and the TORC2 complex, in which mTOR is bound to Rictor. In the TORC1 complex, mTOR signals its downstream effectors, S6 kinase/ribosomal protein and 4EBP-1/eIF-4E, to control protein translation (Inoki et al., 2005). mTOR is generally considered a downstream substrate of Akt, but it can phosphorylate Akt when bound to Rictor in TORC2 complexes (Sarbassov et al., 2005), resulting in positive feedback in the pathway. In addition, the downstream mTOR effector S6 kinase-1 (S6K1) can regulate this pathway by catalyzing the inhibitory phosphorylation of insulin receptor substrate (IRS) proteins, preventing IRS proteins from activating PI3kinase and thereby inhibiting the activation of Akt (Harrington et al., 2004).

4.3 Rational for Targeting the PI3K/Akt Pathway

In addition to preclinical studies, clinical observations support the targeting of the PI3K/Akt/mTOR pathway in human cancers(Vogt et al., 2009). Immunohistochemical studies using antibodies that recognize Akt phosphorylated at S473 have demonstrated that activated Akt is detectable in cancers, including head and neck cancers (Gupta et al., 2002). Moreover, antibodies against S473 and T308, two sites of Akt phosphorylation, showed that Akt was selectively activated in NSCLC compared with normal lung tissue, and that phosphorylation of Akt at both sites was a better predictor of poor prognosis in NSCLC than phosphorylation at S473 alone (Tsurutani et al., 2006). In addition, amplification of Akt isoforms has been observed in some cancers, albeit at a lower frequency. Another frequent genetic event occurring in human cancer is loss of function of the tumor suppressor PTEN, which normally suppresses activation of
the PI3K/Akt/mTOR pathway by functioning as a lipid phosphatase. Loss of PTEN function in cancer can occur through mutation, deletion or epigenetic silencing, with the latter occurring in tumor types, such as lung cancer, in which PTEN mutations are rare (Forgacs et al., 1998). Mutation, deletion or epigenetic silencing of PTEN has been shown to correlate with poor prognosis and reduced survival in patients with various types of cancer (Bertram et al., 2006), with loss of PTEN being a common mechanism for activation of the PI3K/Akt/mTOR pathway and poor prognosis. PI3K activation in human tumors may result from the amplification, over-expression, or mutation of either its p110 catalytic or its p85 regulatory subunit. Amplification of the 3q26 chromosomal region, which contains the PI3KCA gene that encodes the p110α catalytic subunit of PI3K, has been observed in 40% of ovarian and 50% of cervical carcinomas (Ma et al., 2000; Shayesteh et al., 1999). Somatic mutations of this gene have been detected in several cancer types, with kinase activity being greater in mutant than in wild-type PI3K (Samuels & Ericson, 2006). Mutations in the regulatory p85 subunit have also been detected. Any of the alterations in individual components of the PI3K/Akt pathway would result in its activation, and activation of this pathway has been reported to be among the most frequent molecular alterations in tumors (Samuels & Ericson, 2006).

5 Inhibition of PI Synthesis in HNSCC

PIP2, a substrate of PI3Kinase, may be metabolized from PI by two kinases, PI4Kianse and PI5Kinase, indicating the importance of inhibiting the PI metabolic pathway as an anti-tumor strategy. Our laboratory has investigated three potential mechanisms by which inhibition of PI synthesis could affect HNSCCs, anti-proliferation, inhibition of MMP (matrix metalloproteinase) production/activity, and anti-angiogenesis.

a) Anti-proliferation An imbalance between G1 cyclin and CDK (cyclin-dependent kinase) inhibitors (CKIs) has been found to contribute to tumorigenesis and tumor progression. Cyclin D1/PRAD1 acts as a positive regulator of the cell cycle via phosphorylation of pRB (Rb protein) and the formation of a cyclin D1-CDK4 complex, pRB hyperphosphorylated by CDKs releases E2F, which is necessary for the activation of a gene expression network that regulates entry and progression through S phase.

CKIs are classified into two groups: members of the Ink4 family (p15, p16, p18, and p19) for cyclin D/CDK4 or cyclin D/CDK6, and the cip/kip family (p21, p27, and p57) for cyclin D/CDK4 and cyclin E/CDK2. Overexpression of cyclin D1 in HNSCC is an important prognostic marker, predicting sensitivity to chemotherapy and radiotherapy. Furthermore, imbalances between cyclin D1 and its inhibitors, p16 and p27, may be critical for HNSCC development. Strategies to block cyclin D1 function have been studied extensively; for example, the introduction of an antisense cyclin D1 expression vector into cells reduced their growth rate in vitro and decreased tumorigenicity in athymic nude mice (Nakashima & Clayman, 2000). In addition, we found that inhibition of PI synthesis caused G1 arrest of HNSCC accompanied by decreased levels of cyclin D1, cyclin E and phosphorylated pRB (Baba et al., 2001).
b) **Inhibition of MMP production/activity** Tumor metastasis is a complex multistep process, involving tumor growth at the primary site, entry into the circulation (intravasation), adhesion to the basement membranes (BM) of target organs, extravasation and growth at secondary sites. The intravasation and extravasation processes involve degradation of the BM by proteinases, normally MMPs. MMP-9/gelatinase B and MMP-2/gelatinase A are specific for type IV collagen, which acts as the backbone of BM, and therefore probably play a major role in degrading the BM. In HNSCC, MMP-2 and MMP-9 are associated with metastatic potential. Therefore, MMPs are attractive therapy targets and many drugs have been developed to prevent their extracellular matrix-degrading activities during metastasis and angiogenesis. We have demonstrated that inhibition of PI synthesis affects the production of MMP-2 and MMP-9 in HNSCC cell lines (Baba *et al.*, 2000).

c) **Anti-angiogenesis** Angiogenesis, the formation of new blood vessels from pre-existing capillaries or incorporating bone marrow-derived endothelial precursor cells into growing vessels, is associated with the malignant phenotype of cancer. In addition, angiogenesis plays a role in various other diseases, such as diabetic retinopathy, age-related macular degeneration, rheumatoid arthritis, psoriasis, atherosclerosis and restenosis (Cherrington *et al.*, 2000). Clinical association of tumor vascularity with tumor aggressiveness has been demonstrated in a wide variety of tumor types including HNSCC. Therefore, determining microvessel density in tumor tissues can be useful in predicting a patient’s prognosis. Inhibition of angiogenesis can repress the growth rate of tumor cells and lead to cell death resulting from reduced nutrition and oxygen supply to the tumor. VEGF (vascular endothelial growth factor), which plays a major role in many angiogenic processes, binds to its receptor Flk-1/KDR on endothelial cells (EC), stimulating their proliferation through the phospholipase Cγ-protein kinase C-ERK (extracellular signal-regulated kinase) pathway, but not via Ras (Takahashi *et al.*, 1999). In addition, VEGF stimulates EC migration through p38 MAPK (mitogen-activated kinase) independently of ERK (Rousseau *et al.*, 1997). Therefore, these two major MAPK pathways are eligible targets for therapeutic reduction of angiogenesis in HNSCC.

Most clinical trials of anti-angiogenic agents have been conducted in patients with advanced tumors resistant to conventional therapies, with phase III trials comparing the efficacy of an experimental angiogenesis inhibitor plus standard chemotherapy with that of standard chemotherapy alone (Gotink & Verheul, 2010). Several recent clinical trials have shown that blocking VEGF signaling had significant clinical benefit (Ho & Kuo, 2007). SU11248, a tyrosine kinase inhibitor of the Flk-1/KDR receptor (VEGF receptor), and bevacizumab, a monoclonal antibody to VEGF, have been approved by the FDA (Ho & Kuo, 2007). Furthermore, we have demonstrated that inhibition of PI abrogated VEGF stimulation of the growth and migration of human umbilical vein ECs through the ERK-cyclin D1 and p38 pathways, respectively (Baba *et al.*, 2004). Increased PI synthase expression is an early event in HNSCC (Kaur *et al.*, 2010), so inhibition of PI synthesis may be a potent therapeutic strategy in patients with these tumors (Baba *et al.*, 2010).
6 Cross Talk with respect to the PI3K/Akt Pathway

6.1 Cross Talk between EGFR and IGF1R

Growth factor switching from one pathway to another may be an adaptive mechanism, induced by blocking the dominant growth factor receptor pathway. Blockade of EGFR signaling in DU145 and PC-3 human prostate cancer cells has found to enhance the growth promoting effects of the peptide growth factor ligands basic fibroblast growth factor (bFGF) and IGF-1, respectively (Jones et al., 1997). More recently, the EGFR-selective tyrosine kinase inhibitor gefitinib has been shown to inhibit the growth of EGFR-positive MCF-7-derived tamoxifen-resistant breast cancer cells, an effect that can be abrogated by exposing the cells to non-EGF ligands such as heregulin-β and IGF-II (Knowlden et al., 2005). The reversal of the anti-tumor effects of gefitinib by IGF-II, acting through IGF-1R, is accompanied by reactivation of the previously reduced activity of Akt and extracellular-regulated kinase (ERK), with ERK signaling contributing to the re-establishment of tumor cell growth. Therefore, in the presence of a dominant growth pathway, cancer cells are capable of responding to other growth factors, compromising the anti-tumor activity of agents designed specifically to inhibit EGFR. Importantly, blockade of EGFR signaling frequently results in switching to the IGF-1R pathway, a common mechanism used to promote resistance to anti-EGFR treatment (Choi et al., 2010). For example, gefitinib initially inhibited the growth of the EGFR-positive DU145 prostate cancer cell line and of MCF-7-derived tamoxifen- and fulvestrant-resistant breast cancer cell lines, but chronic exposure to gefitinib resulted in the development of gefitinib-resistant variant sub-lines, all showing up-regulation of multiple IGF-1R signaling components when compared with their parental cell lines (Jones et al., 2004). This resulted in increased production and elevated expression of the IGF-1R ligand IGF-II, increased activity of IGF-1R and increased levels of Akt activity. In addition, although the A549 lung cancer cell line is partially sensitive to gefitinib, chronic exposure resulted in a resistant variant with increased activity of elements of the IGF-1R pathway. The importance of IGF-1R signaling in cell lines with acquired gefitinib resistance was supported by the increased dependency of these cell lines on IGF-1R signaling and their greater sensitivity to growth inhibition by IGF-1R-selective TKIs (Jones et al., 2004). Therefore, the dominance of the EGFR pathway in parental cells was replaced by an increased use of the IGF-1R pathway in gefitinib resistant cells.

Growth factor pathway switching may result not only from changes occurring during the development of acquired resistance, but, critically, may occur rapidly and modulate initial sensitivity to EGFR-blockade, resulting in de novo or intrinsic resistance to anti-EGFR agents such as gefitinib. Indeed, although the EGFR and IGF-1R pathways are classically regarded as separate entities, the overlapping of downstream signal transduction molecules indicates that these receptors can affect each other’s signaling abilities, although the precise mechanisms involved in this crosstalk have not been fully elucidated. For example, gefitinib only partially blocks EGFR activity in A549 lung cancer cells, accompanied by a dramatic increase in the activity but not the expression of IGF-1R. Moreover, in these cells, IGF-1R can transphosphorylate EGFR, maintaining EGFR activity in the presence of gefitinib. Therefore, by enhancing IGF-1R activity, gefitinib limits its own efficacy in these cells. Interestingly, gefitinib was observed to enhance insulin receptor activity and levels of downstream activated Akt in de novo gefitinib-resistant LoVo colorectal cancer cells, which are defective in their ability to produce mature IGF-1R and predominantly express insulin receptor-isoform A (InsR-A), a member of the IGF-1R family (Jones et al., 2006). Furthermore, InsR can modulate and maintain EGFR phosphorylation in these cells. Such rapid and dynamic interplay between EGFR and IGF-1R or InsR may play an important role in limiting the anti-tumor
activity of gefitinib; partial and de novo resistance to this inhibitor has been demonstrated in A549 and LoVo cells, respectively.

Treatment of HNSCC cells and xenografts with combinations of antibodies to IGF-1R and EGFR was more effective than either agent alone at reducing cancer cell growth (Barnes et al., 2007), suggesting that these combined anti-tyrosine kinase receptor directed therapies may have benefit in treating patients with HNSCC. Similarly, treatment with small molecules targeting these two pathways suppressed the growth of HNSCC cells (Slomiany et al., 2007), as did the combination of cetuximab with a PI3K inhibitor (Rebucci et al., 2011).

6.2 Cross Talk between EGFR and c-MET

The transmembrane receptor tyrosine kinase MET was shown to contribute to resistance to EGFR inhibitors in cell lines derived from head and neck, breast, gastric and lung cancers as well as in lung samples. In one study, MET amplification was detected in 9 of 43 (21%) lung tumors with acquired resistance to EGFR inhibitors, but in only 2 of 62 (3%) lung tumors with EGFR mutations from patients not previously treated with kinase inhibitors (Bean et al., 2007). MET amplification causes gefitinib resistance by driving ERBB-3 dependent phosphorylation and by maintaining persistent activation of PI3K/Akt signaling in the presence of EGFR inhibition (Engelman et al., 2007). In the absence of MET amplification, MET signaling can be activated by increased expression of the MET ligand hepatocyte growth factor (HGF), leading to resistance to EGFR TKIs in lung adenocarcinoma patients harboring EGFR-activating mutations, again associated with persistent PI3K/AKT activation (Yano et al., 2008). Cortactin, a regulator of dynamic actin networks, was shown to regulate MET signaling and promote resistance to EGFR inhibitors in HNSCC cell lines (Yano et al., 2008). Overexpression of cortactin in these cells stabilized MET, enhanced HGF-mediated mitogenesis and activated Akt, leading to resistance to gefitinib. Interestingly, the gene encoding cortactin, CTNN, resides at a chromosomal locus (11q13) frequently amplified in head and neck cancers.

6.3 Cross Talk between EGFR and VEGFR

Tumor-induced angiogenesis plays an important role in cancer development and has been linked to EGFR resistance. EGF activation of EGFR signaling results in the secretion of proangiogenic growth factors such as VEGF, whereas blockade of EGFR inhibits the secretion of VEGF. Continuous treatment of xenograft tumors with anti-EGFR antibody resulted in the generation of six A431 human squamous cell carcinoma cell lines resistant to EGFR inhibition (Viloria-Petit et al., 2001), with five of these cell lines showing elevated VEGF expression, increased angiogenic potential in vitro and increased tumor angiogenesis in vivo. Furthermore, A431 cells genetically engineered to overexpress VEGF displayed resistance to anti-EGFR antibodies in vivo (Viloria-Petit et al., 2001). Thus, resistance to EGFR antagonists may well be mediated via VEGF signaling.

Tumor-induced angiogenesis may be involved in the development and progression of HNSCC. A meta-analysis of 12 studies of VEGF protein overexpression and clinical outcome in patients diagnosed with HNSCC found that the overall risk of death within 2 years was approximately 1.9-fold higher in patients with VEGF-positive than VEGF-negative tumors (Kyzas, et al., 2005). The co-overexpression of VEGF and VEGF receptor2 (VEGFR2) was correlated with a higher tumor proliferation rate and shorter survival in patients with HNSCC (Kyzas, Stefanou, et al., 2005). The cross-talk between the VEGFR and EGFR signaling cascades in the biological context of HNSCC has not been fully characterized; however, ligand-VEGFR interaction activates the tyrosine kinase domain of the VEGFR, activating intracellular
signaling transduction pathways, such as the PI3K/Akt pathway, involved in regulating cellular survival. Because HNSCC tumor cells also express VEGFR (Lalla et al., 2003), there may be cross talk between EGFR and VEGFR through the PI3K/Akt pathway in HNSCCs, and ongoing clinical studies are exploring the combination of anti-VEGF and anti-EGFR therapies in patients with these tumors.

7 Cross Talk with respect to STAT3 Signaling Pathways

7.1 Cross Talk between EGFR and Gp130 Receptor

Although EGFR activation has been found to lead to the rapid phosphorylation of STAT3 on tyrosine 705 and the subsequent activation of STAT3-dependent gene expression, STAT3 tyrosine phosphorylation and the formation of active STAT3 DNA-binding complexes were insensitive to EGFR inhibition in many HNSCC cell lines (Sriuranpong et al., 2003). Indeed, of a representative panel of 10 HNSCC-derived cell lines, 9 showed increased tyrosine phosphorylation and STAT3 activity, but only 3 showed constitutive activation of EGFR (Sriuranpong et al., 2003). In searching for the mechanism responsible for the EGFR-independent activation of STAT3 in HNSCC cells, the activation of the gp130 cytokine receptor subunit was found to promote the phosphorylation of STAT3 at tyrosine 705 through the activation of intracellular tyrosine kinases of the JAK family. Surprisingly, gp130 activation was found to be initiated primarily by interleukin (IL)-6, a cytokine secreted by HNSCC cells that binds to the cell surface in an autocrine fashion. These findings suggest that the persistent activation of STAT3 in HNSCC can result from the deregulation of EGFR activity or from the EGF-independent autocrine activation of STAT3 by tumor-secreted cytokines. Furthermore, overexpression of IL-6 in HNSCC cells was found to involve increased transcription from the IL-6 promoter, which is dependent on the presence of an intact NFkB response element located 63 to 75 bp upstream of the IL-6 transcriptional initiation site. Inhibition of NFkB resulted in the marked downregulation of IL-6 mRNA and protein expression, concomitant with the decreased release of other inflammatory cytokines, such as IL-8, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF). Surprisingly, the blockade of NFkB also resulted in the drastic inhibition of constitutive STAT3 activity in HNSCC cells, as reflected by the reduced tyrosine phosphorylation of STAT, and by blockade of the autocrine/paracrine activation of STAT3 (Squarize et al., 2006). These findings are indicative of cross-talk between the NFkB and STAT3 signaling systems, a cross-talk initiated by the release of IL-6, which, in turn, results from the NFkB-dependent activation of the IL-6 promoter and the subsequent tyrosine phosphorylation of STAT3 by the autocrine/paracrine activation of IL-6 receptors in tumor cells.

7.2 Cross Talk between EGFR and c-MET

MET signaling has been shown to contribute to intrinsic resistance to EGFR tyrosine kinase inhibitors in breast cancer; MET activated c-Src in a resistant breast cancer cell line, leading to tyrosine phosphorylation of EGFR even in the presence of EGFR TKIs (Mueller et al., 2008). STAT3 is phosphorylated by EGFR, EGFR-interactor Src, and c-MET, and phosphorylated STAT3 translocates to the nucleus and activates the transcription of genes involved in cell cycle progression, angiogenesis, and apoptotic resistance.
8 Cross Talk with respect to ERK Signaling Pathways

8.1 Cross Talk between EGFR and c-MET

EGFR and c-MET drive cellular proliferation by activating the Ras-Raf-MEK-ERK pathway, because c-MET is highly expressed in HNSCC, cross talk between EGFR and c-MET may occur through the ERK pathway.

8.2 Cross Talk between EGFR and IGF-1R

IGF-1R may stimulate cell proliferation by activating the ERK pathway, inducing resistance to EGFR inhibitors (Ahmad et al., 2004; Morgillo et al., 2006). This novel function of IGF-1R has been validated in NSCLC cell lines (Morgillo et al., 2006) and most recently in an HNSCC model (Barnes et al., 2007). Stimulation of HNSCC cell lines with IGF resulted in the heterodimerization of IGFR with EGFR, with activating phosphorylation of both receptors (Barnes et al., 2007). Furthermore, treatment with the combination of an anti-IGF-1R therapeutic antibody, A12, and the anti-EGFR antibody cetuximab more effectively inhibited cellular proliferation and migration than treatment with either alone. The ability of this drug combination to inhibit EGFR-resistant HNSCCs in the preclinical and clinical settings remains to be determined.

8.3 Cross Talk between EGFR and VEGFR

The cross-talk between the EGFR and VEGFR signaling cascades in HNSCC has not been fully characterized. However, HNSCC cells express VEGFR. Because VEGF stimulates cell proliferation through the phospholipase Cγ-protein kinase C-ERK pathway, but not via Ras (Takahashi et al., 1999), there may be cross talk between EGFR and VEGFR through the ERK pathway.

9 Future Prospects

Signaling of multiple receptor tyrosine kinases (RTKs) is propagated through Akt. Therefore, simultaneous inhibition of EGFR and pathway components such as Akt and mTOR could circumvent the feedback activation observed with either approach alone. The most extensive data concerning proximal and distal signaling inhibition has been observed by combining PI3K/Akt/mTOR pathway inhibitors with EGFR antagonists. Several PI3K inhibitors can restore cellular sensitivity to EGFR inhibitors. For example, the selective PI3K inhibitor PX-866 and p110α were found to abrogate gefitinib resistance in NSCLC xenografts (Ihle et al., 2005). Synergistic effects of rapamycin and EGFR TKIs have been observed in several in vitro systems, including glioblastoma multiforme, prostate cancer, pancreatic cancer, squamous cell carcinoma, renal cell carcinoma, leukemia, cervical carcinoma and NSCLC cell lines, as well as in some xenografts (Birle & Hedley, 2006; Buck et al., 2006; Costa et al., 2007; Hjelmeland et al., 2007; Jimeno et al., 2007; Mohi et al., 2004). The combination of rapamycin and erlotinib showed re-sensitization and synergistic growth inhibition in cell lines that were previously resistant to erlotinib (Buck et al., 2006). Moreover, the combination of rapamycin and the irreversible EGFR TKI, HKI-272, resulted in the significant regression of lung tumors in transgenic mice possessing the secondary resistance mutation T790M (Li et al., 2007). Addition of the dual PI3K/mTOR inhibitor PI-103 to erlotinib was necessary to induce growth arrest of human glioma cell lines with mutant PTEN (Fan et al., 2007), suggesting that activation
of the PI3K/Akt/mTOR pathway by EGFR-independent mechanisms confers resistance to EGFR inhibitors, but that this resistance can be overcome by the addition of pathway inhibitors. Collectively, these findings suggest that the combination of EGFR antagonists and PI3K/Akt pathway inhibitors may be beneficial to patients with tumors resistant to EGFR TKIs. These combinations, however, may be insufficient for the treatment of some patients with HNSCC, due to the cross talk between the ERK and STAT3 signaling pathways, as described in sections 7 and 8, and Figure 1. Because intracellular signaling of EGFR occurs via various pathways, including those involving ERK, PI3K/Akt, and STAT3, EGF-dependent induction of anti-apoptotic proteins and cell cycle inhibitors is highly variable in HNSCC cells (Rüddel et al., 2010). Therefore, personalized therapy with combinations of EGFR antagonists and PI3K/Akt/mTOR inhibitors, ERK, and STAT3 pathway inhibitors is needed.

**Figure 1:** Proposed mechanism for overcoming HNSCC resistance to EGFR antagonists using PI3kinase/Akt/mTOR, STAT3 and ERK pathway inhibitors. There is molecular cross-talk between EGFR and signaling via other RTKs, including gp130, IGF1R, c-MET, and VEGFR, through PI3K/Akt and STAT3. Furthermore, in HNSCCs, molecular cross-talk between the EGFR and other RTK signaling pathways, such as IGF1R, c-MET, and VEGFR, can occur through ERK.

**10 Conclusions**

EGFR is expressed at a high level in HNSCC, but EGFR inhibitor monotherapy has had limited success in patients with these tumors. EGFR mutations are extremely rare in HNSCC. Three major signaling pathways have been found to mediate the downstream effects of EGFR: the PI3K/Akt, STAT3, and ERK pathways. The PI3K/Akt and STAT3 pathways are responsible for cellular survival and there is molecular cross-talk between EGFR and other RTKs that signal through PI3K/Akt and STAT3 in HNSCCs. Furthermore, the ERK pathway is responsible for cell proliferation, and there is molecular cross-talk between the EGFR and other RTK signaling pathways through ERK in HNSCCs. Hence, EGFR inhibitors alone
may be unable to suppress EGFR downstream, and may affect ADCC activity (Kondo et al., 2011). Therefore, personalized combination therapy targeting these signaling pathways, PI3K/Akt, STAT3, ERK, and EGFR, may provide clinical benefits for patients with HNSCC.

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Conflicts of Interests
The authors disclose no conflict of interests.

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