Chapter

Integrated Molecular Profiling as an Approach to Identify PI3K Inhibitor Resistance Mechanisms

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Abstract

The identification of drug resistance pathways and approaches to target these pathways remains a significant and important challenge in cancer biology. Here, we address this challenge in the context of ongoing efforts to advance phosphatidylinositol 3-kinase (PI3K) inhibitors for the treatment of PI3K-aberrant cancers. While PI3K inhibitors have had tremendous success in some diseases, such as breast cancer, early clinical trials in other malignancies, such as head and neck squamous cell carcinoma (HNSCC), have not had the same level of success. Since HNSCC and other cancers display relatively high PI3K pathway alteration rates (>45%), these underwhelming results suggest that additional or unexpected factors may contribute to the lower response rates. Here, we highlight some of the emerging functional genomic and sequencing approaches being used to identify predictive biomarkers of PI3K inhibitor response using both cancer cell lines and clinical trial specimens. Importantly, these approaches have uncovered both innate genetic and adaptive mechanisms driving PI3K inhibitor resistance. In this chapter, we describe recent technological advances that have revolutionized our understanding of PI3K inhibitor resistance pathways in HNSCC and highlight how these and other approaches lay the groundwork to make significant strides in our understanding of molecular pharmacology in the cancer field.

Keywords: PI3K, targeted therapy, drug combination, drug screening, drug resistance

1. Introduction

Head and neck squamous cell carcinomas (HNSCCs) are malignant neoplasms that can occur in regions including the oral cavity, oropharynx, and larynx. HNSCC is the sixth most common cancer, by incidence, worldwide and constitutes approximately 4% of all cancers globally [1–3]. Tobacco use, alcohol consumption, and/or infection with oncogenic high-risk types of HPV, primarily HPV16, are regarded as the major risk factors for HNSCC [4]. Although traditional treatments for HNSCC include surgery, radiotherapy, and cytotoxic chemotherapy [1], these approaches have only modestly reduced the mortality of HNSCC. In fact, only 40–50% of patients with HNSCC survive for 5 years following diagnosis [5].

2. The phosphatidylinositol 3-kinase (PI3K) pathway in HNSCC

HNSCC sequencing studies have identified highly prevalent PI3K pathway alterations that activate PI3K signaling. Following activation by receptor tyrosine kinases (RTKs) and/or G-protein coupled receptors (GPCRs), PI3K phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP_2) into an essential second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) [6]. PIP_3 then recruits and activates proteins like PDK1 and AKT to mediate PI3K's pro-survival functions (**Figure 1A**). As a tumor suppressor, PTEN dephosphorylates PIP_3 into PIP_2 to prevent downstream signal propagation; the deregulation of PTEN is also related to multiple cancers including HNSCC [7].

There are three classes of PI3K (Class I, Class II, and Class III), and Class I PI3K is further divided into Class IA and Class IB [8]. Among the Class IA PI3Ks is p110alpha encoded by the gene *PIK3CA*, which represents the catalytic subunit and alpha isoform of PI3K. Importantly, *PIK3CA* is the most frequently altered gene in the PI3K pathway across all tumors and in HNSCC [9–11]. Alterations of other Class I PI3Ks isoforms, like p110β, and of some regulatory isoforms have been detected in various cancers, albeit with a relatively low frequency [12] (**Figure 2**). Although studies have also examined Class II and Class III PI3Ks, more research is needed to understand their role in human disease [16].

Functionally, the PI3K signaling pathway has a wide range of intracellular effects, including participation in cell cycle, survival, metabolism, motility, and genomic instability [17]. Mutations or other genetic aberrations can lead to hyperactivation of PI3K signaling, and in turn increase cell growth and viability. Angiogenesis and inflammatory cell recruitment, which are thought to be cancer-promoting, may also drive tumor progression and are common in advanced-stage tumors [9, 18].

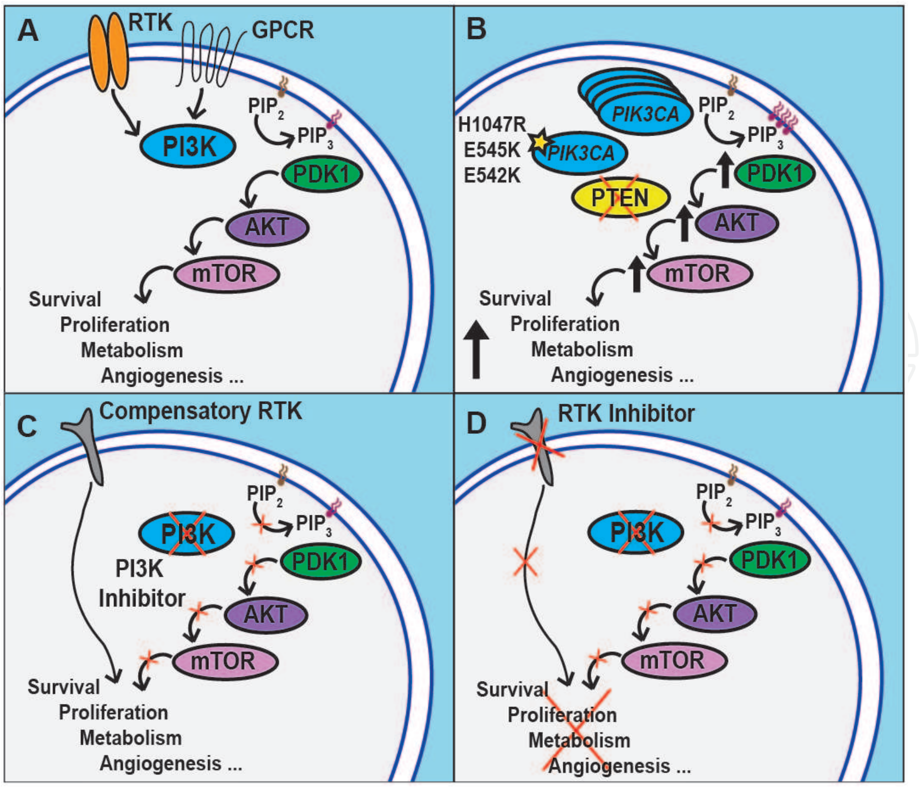

Figure 1.
Rationale for PI3K inhibitor combination therapy. (A) The PI3K signaling pathway has diverse cellular functions. (B) Alterations in PI3K pathway genes may lead to increased signaling. (C) Resistance to PI3K inhibitor monotherapy prevents cell death. (D) Co-targeting PI3K and resistance mechanisms leads to cell death.

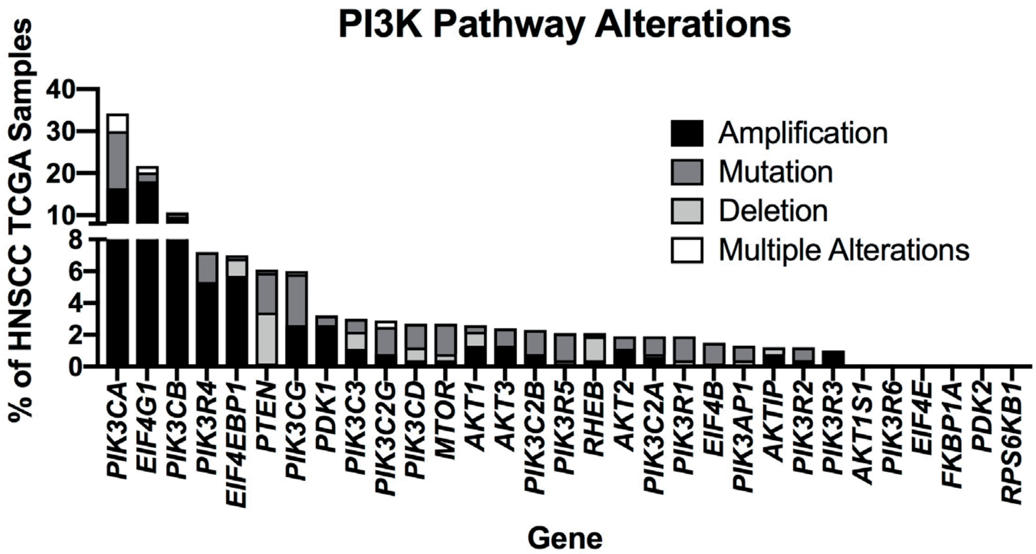

Figure 2.
PI3K pathway gene alteration rates in HNSCC tumors from the cancer genome atlas (TCGA). Amplifications, deletions, mutations and/or multiple alterations (e.g., amplification and mutation) are shown as indicated [13–15].

The first manuscript detailing *PIK3CA* mutations in HNSCC was published in 2006; this study described "hotspot," activating mutations (E542K, E545K, and H1047R) among other *PIK3CA* alterations that are less frequent and have not been as well characterized [19]. Since then, evidence has continued to support the significant role of PI3K signaling in HNSCC. The Cancer Genome Atlas (TCGA) dataset, one of the largest HNSCC sequencing studies performed to date, shows that the PI3K signaling pathway is the most frequently mutated oncogenic and targetable signaling pathway in this cancer type [13, 15, 20]. Additionally, Chung and co-authors independently found that almost 32% of HNSCC patients have *PIK3CA* mutation or copy number alteration after analyzing 252 HNSCC patient samples. This study also indicated that 11% of HPV-positive and 5% of HPV-negative HNSCC patients have loss-of-function mutations or copy number alterations in *PTEN*, the gene responsible for restraining PI3K pathway activation (**Figure 1B**) [21].

The frequency of PI3K pathway alteration suggests that inhibitors targeting this pathway may be of clinical use, and several teams have advanced PI3K inhibitors to test their effectiveness for HNSCC treatment. Early clinical trials demonstrated that PI3K inhibitors were safe for use in patients with solid tumors [22, 23], and studies evaluated the potential benefits of using pan-PI3K-targeting agents in recurrent and metastatic HNSCC specifically. However, PI3K inhibitors have more recently shown limitations in efficacy as well as safety (**Figure 1C**). Jimeno et al. reported in 2015 that pan-PI3K inhibitor PX-866 did not improve outcomes when added to cytotoxic chemotherapy (docetaxel) in unselected recurrent or metastatic (R/M) HNSCC patients [24]. Later, Soulieres and colleagues achieved improvements in overall and progression-free survival when administering another pan-PI3K inhibitor, BKM120, with cytotoxic agent paclitaxel (as compared to paclitaxel alone); this study, the BERIL-1 trial, is discussed further below [25]. Unfortunately, BKM120 has not been evaluated further due to undesired side effects. Current trials are evaluating the clinical effects of other PI3K-targeting drugs (NCT03740100), including those with isoform selectivity (NCT02145312, NCT02822482), in HNSCC patients.

The lack of patient selection is one potential contributor to the underwhelming efficacy of PI3K inhibitor treatment in HNSCCs to date. Although the majority of TCGA HNSCC patients display aberration in one or more PI3K pathway genes [14], the status of any single gene or any group of genes has not been validated as a critical biomarker of response to PI3K inhibition. This is in contrast to recent data for hormone receptor-positive breast cancer, where PI3K inhibitor alpelisib is used in combination with fulvestrant to prolong survival for patients with *PIK3CA* mutant tumors [26]. While precision medicine trials across a variety of cancer types have also shown a trend supporting *PIK3CA* alterations as a marker for response to PI3K inhibitors [27], *PIK3CA* mutation has not yet been associated with sensitivity to PI3K inhibition in HNSCC trials [24, 28]. Studies evaluating the role of *PIK3CA* mutations in response to PI3K inhibition in HNSCC often do not reach statistical significance or have a very limited number of patients enrolling. For example, Janku et al. conducted an analysis of responses to PI3K/AKT/mTOR inhibitors in tumors with H1047R *PIK3CA* mutations including four HNSCC patients; after inhibitor treatment, two of these patients experienced progressive disease, one had little change in tumor burden, and another had an incomplete response to therapy [29]. It is possible that the difference in outcomes between tumors with and without *PIK3CA* mutations has not been noted due to an insufficient number of *PIK3CA* mutant tumors in any single clinical trial. Ongoing studies of PI3K inhibitor copanlisib in HNSCC patients with *PIK3CA* mutation or amplification or *PTEN* loss will better elucidate any potential differences in response attributable to PI3K activation (NCT02822482). It is also feasible that other features predict responses to PI3K inhibitor. In the recent BERIL-1 study, which compared outcomes in R/M HNSCC patients (n = 79 per group) treated with paclitaxel with or without pan-PI3K inhibitor BKM120, follow-up analysis revealed that *TP53* alteration, low tumor mutation burden, HPV negativity, and high infiltration of tumor infiltrating lymphocytes (TILs) or CD8+ T-cells was associated with improved response to BKM120. Recent preclinical work has proposed that loss-of-function mutations in *NOTCH1* may predict response to PI3K inhibition [30]. Together these data suggest that a more nuanced understanding of tissue type-specific factors and PI3K inhibitor response mechanisms may be required to develop clinically effective companion diagnostics for this class of inhibitors in HNSCC.

Our recent report indicates that responses to PI3K inhibition, either as monotherapy or in combination with other targeted inhibitors, are complex and cannot be predicted solely based on genetic mutation, copy number alteration, or RNA expression of a single gene (submitted). In this study, a diverse set of HNSCC cell lines were treated with PI3K inhibitors of varying isoform selectivity as monotherapies. Alpha isoform-targeting agents were clearly more effective than other PI3K inhibitors, but the sensitivity profiles for any individual pan- or alpha-isoform PI3K inhibitor were more difficult to stratify. This was increasingly true when PI3K inhibitors were used in drug combinations: *PIK3CA* mutation, copy number, RNA expression, and HPV status did not prove to be meaningful biomarkers for either PI3K and epidermal growth factor receptor (EGFR) inhibitor dual-therapies [31] or for other synergistic drug pairs. In the case of PI3Kalpha inhibitor HS-173 and FAK inhibitor TAE226, greater synergy was observed in *PIK3CA* mutant cell lines as compared to *PIK3CA* wildtype cell lines in initial validation experiments. However, when this association was tested more rigorously with other PI3K and FAK inhibitors, dual-therapy was beneficial also in many *PIK3CA* wildtype models; this could be due to differences in selectivity or mechanism of action for individual small molecule drugs [32]. Thus, PI3K pathway activation, measured at the DNA level via mutation or copy number status or at the protein level via relative downstream phosphorylation, appears to be an insufficient biomarker for sensitivity in HNSCC cell lines; other cellular features, including additional alteration in PI3K pathway members (such as downstream mutations in *AKT1*) or activation of receptor tyrosine kinases (perhaps via upstream overexpression of EGFR), may contribute to signaling through the PI3K pathway and thereby affect inhibitor responses. Indeed, multifaceted analyses, such as those considering gene sets rather than individual genetic changes, may be needed to predict sensitivity. For example, responses to EGFR or FAK inhibitor may be better stratified using gene sets incorporating activation of PI3K, mTOR, or other related signaling nodes instead of *PIK3CA* mutation status alone. Alternatively, an additional pathway that is changed as a result of PI3K activation (e.g., epithelial to mesenchymal transition, cell cycle, or apoptosis) may be even more effective in predicting response.

More broadly, activation or blockade of PI3K signaling may impact response to other forms of cancer treatment. Clinical trial data have not demonstrated that mutation, amplification, or loss of PI3K pathway genes is linked to sensitivity or resistance to PI3K inhibitors. However, recent trials for EGFR-targeting agents have noted poorer outcomes following EGFR inhibition in patients with PI3K activation. In the phase III E2303 trial, which compared cisplatin with or without cetuximab in without cetuximab in R/M HNSCC patients [33], *PIK3CA* mutation or *PTEN* loss was associated with poor response to EGFR-targeting therapy [34]. This finding was also noted in the LUX-H&N1 trial, which compared second-line treatments with afatinib and methotrexate in R/M HNSCCs [35]; here, tumors lacking *PTEN* received inferior benefit from afatinib as compared to those with high levels of *PTEN* [36]. Preclinical studies have also noted that PI3K activation or *PTEN* loss may serve as a biomarker for resistance to cetuximab [37, 38] and that *PIK3CA* mutant HNSCC models may be more resistant to cyclin-dependent kinase (CDK) inhibitor palbociclib [39]. Further studies are warranted to validate these results in larger, prospective trials.

3. Combination drug strategies may overcome compensatory PI3K inhibitor resistance

3.1 Epidermal growth factor receptor (EGFR)

One of the most widely studied mechanisms of resistance to PI3K inhibition is signaling through members of the ERBB family, primarily EGFR, and the downstream Ras-MEK-ERK effector pathway. The human ERBB gene family encodes four members of the ERBB family of receptors including EGFR/ERBB1/HER1, NEU/ERBB2/HER2, ERBB3/HER3 and ERBB4/HER4. As a transmembrane receptor, EGFR can be activated by ligands including epidermal growth factor (EGF). EGFR signaling subsequently activates downstream pathways, including RAS-RAF-MEK-ERK. MAPK and PI3K-AKT-mTOR signaling pathways, to promote cell proliferation and inhibit apoptosis in many head and neck models [40, 41]. EGFR overexpression has been reported in the vast majority of HNSCCs [42]. Further genetic dysregulations of EGFR signaling caused by *EGFR* gene mutation (although rare in head and neck cancer) and *EGFR* gene copy number amplification (approximately 10% of cases) also contribute to activated EGFR signaling [41, 43].

Activation of EGFR signaling as a mechanism of PI3K inhibitor resistance has been extensively characterized using a wide variety of cell lines (displaying a diverse array of genetic alterations) and a large set of ERBB family-targeting drugs [1, 31, 44–47]. Early work focused on *PIK3CA*-amplified HNSCCs demonstrated that two-thirds (67%) of cell lines with additional copies of wildtype *PIK3CA* maintained RAS-RAF-MEK-ERK MAPK pathway activity following PI3K inhibitor treatment and that two of these models were also sensitive to dual inhibition of PI3K and EGFR or MEK [1]. A more recent publication extended this observation to a larger panel of ERBB inhibitors and cell lines, including several with *PIK3CA* mutations [31]. Overall, findings from the latter study mirrored those of previous publications showing that dual-therapy with PI3K and EGFR inhibitors was often more effective than either monotherapy [44–47], but also extended this observation to consider individual classes of ERBB-targeting agents that might result in heightened responses when used as part of combination treatments. Results in HNSCC cell lines showed that *irreversible* inhibitors of EGFR were more effective in combination with PI3K inhibitors than reversible ERBB-targeting agents. As previous work had primarily considered dual-therapies that included either reversible EGFR inhibitors or EGFR-targeting antibodies such as cetuximab, this work was the first direct comparison of PI3K inhibitors in combination with distinct pharmacologies against EGFR.

Beyond direct inhibition of the receptor tyrosine kinases PI3K and EGFR themselves, previous work has also examined drug combinations targeting PI3K and EGFR via inhibition of downstream effectors including mTOR and MEK, respectively. Several papers have described synergy with mTOR inhibitors and EGFR agents [48–50]. In one of these studies, Jimeno et al. used H1047R *PIK3CA* mutant Detroit 562 cells in a xenograft model and noted improved response to mTOR inhibitor temsirolimus and erlotinib. This response co-occurred with changes in MAPK and p70 S6 kinase phosphorylation (downstream of EGFR and mTOR, respectively) and in Ki67, effects that were not evidenced in less responsive xenograft models or after single-agent treatment. Other work with Detroit 562 in vitro showed minimal responses to PI3K inhibitor HS-173 and reversible EGFR inhibitors, including erlotinib, that could be enhanced to synergistic levels with multiple irreversible EGFR inhibitors [31]. The combination of erlotinib with mTOR inhibitors (temsirolimus or otherwise) has not been reported in this model. Nevertheless, since additional data show that ineffective reversible EGFR inhibitor combinations block MAPK phosphorylation [31] (p70 S6 kinase phosphorylation was not tested), it is possible that one or more additional effectors, perhaps further downstream of MAPK/p70 S6 kinase or as part of a second escape pathway, may be responsible for synergistic effects. Alternatively, in vitro and in vivo responses to mTOR and EGFR agents in Detroit 562 and potentially other HNSCCs could be dependent on distinct mechanisms. PI3K and irreversible EGFR inhibitor combinations have not yet been tested in xenografts, but these experiments would enhance our understanding of the potential for such dual-therapies to translate clinically.

In light of the synergy observed following treatment with agents targeting the PI3K and EGFR pathways in preclinical models, phase I and II trials have been performed to examine these dual-therapies in HNSCC patients. Of these trials, three have been completed, all in patients receiving second-line treatment due to chemotherapy resistance, recurrence, and/or metastasis. The first of these trials examined temsirolimus with cetuximab and resulted in dose-limited toxicities in one-third of patients [51]. The second considered another mTOR inhibitor, everolimus, with erlotinib. This combination had a reasonable toxicity profile and stopped or decreased tumor growth in several patients, but it did not result in clinical benefit as compared to previous trials considering erlotinib as a monotherapy [52]. The third trial, which considered cetuximab with or without PI3K inhibitor PX-866, also did not provide evidence of improvement with the addition of PI3K inhibitor [53]. Several other trials using PI3K- and EGFR-targeting agents, sometimes alongside cytotoxic chemotherapy or radiotherapy, have been initiated and are in various stages of completion. Toxicity seems to be a major concern in many of these studies and may limit the use of such combinations in patients. As a result, the development of more specific combinations is warranted.

Nevertheless, previous work also suggests that the use of currently available PI3K and EGFR therapies may be optimized in other ways. For example, the sequence of combination treatments may be an important consideration. Lattanzio et al. showed that Cal-33 cells responded to treatment with EGFR antibody followed by PI3K inhibitor [54], while other data show minimal responses in this model when EGFR- and PI3K-targeting small molecules were co-administered [31]. The type of EGFR-targeting agent that was used may also explain this conflicting data: small molecules (e.g., gefitinib, erlotinib, afatinib) and biologics (e.g., cetuximab) could have very different response profiles in combination with PI3K inhibitors.

Overall, although responses to PI3K and EGFR inhibitor combinations in HNSCC have been studied more extensively than many other dual-therapies for this cancer type, current results highlight the diversity of responses to agents targeting these two signaling pathways. Additional factors, including but not limited to timing, patient selection, and other co-treatments, require further consideration before compensation through the PI3K and EGFR pathways will be most effectively exploited in a population of HNSCC patients.

3.2 Other ERBB family members

Additional studies have successfully implicated other means of upstream inhibition in combination with PI3K-targeting drugs to improve responses in HNSCC, including other members of the ERBB family. While the structure of these members is similar to that of EGFR, each ERBB receptor has a different ligand binding specificity and physiological role [55]. Abnormalities of ERBB2, ERBB3, and ERBB4 have been reported in various malignancies such as breast cancer and occur in smaller subsets of head and neck cancer; therefore, they also became the targets for cancer therapies and have been considered in drug combinations [55, 56].

For example, Brand and co-authors blocked ERBB3 signaling as a means of reversing PI3K inhibitor resistance mediated by HPV oncoproteins E6 and E7 in HPV-positive HNSCCs [57]. These studies support the role of RTKs in HNSCC resistance mechanisms and validate that combination PI3K and RTK blockade may result in improved PI3K inhibitor responses. Likewise, Meister et al. also reported that PI3K inhibition induces ERBB3 upregulation and activation while the combination of PI3K and ERBB3 inhibition has synergistic effect, suppressing HNSCC growth both in vitro and in vivo [58]. Furthermore, in breast cancer, researchers also found that amplification of *ERBB2* can activate PI3K-AKT signaling directly and indirectly [59], and ERBB2 inhibition potentiates the antitumor effect of PI3K inhibitor BEZ235 in this cancer type [60].

3.3 AXL receptor tyrosine kinase

PI3K inhibitor resistance mechanisms, of course, span far beyond the ERBB family of RTKs alone. For instance, the AXL receptor tyrosine kinase is highly expressed in various cancers including esophageal squamous cell carcinoma and HNSCC [61]. Based on work by Elkabets et al., AXL is upregulated particularly in PI3K inhibitor-resistant HNSCCs [62]. These researchers also showed that the dimerization of AXL and EGFR promotes signaling through PI3K and mTOR by activating the PLCγ-PKC signaling pathway, thereby limiting the efficacy of PI3K inhibitors. This PI3K-independent activation of mTOR counteracts the growth inhibitory effect of PI3K inhibition and mediates drug resistance in some head and neck models [62]. Further, Badarni et al. chose to identify and target the transcription factors that were responsible for the increased expression of AXL in HNSCCs. In doing so, they discovered that a c-JUN, a member of the AP-1 transcription factor (TF) complex, was likely responsible for AXL upregulation and that blocking this TF improved the response to PI3K inhibitor BYL719 [63].

3.4 Insulin-like growth factor-1 receptor (IGF-1R)

IGFR signaling is vital in the development of tissues [64] and is aberrantly implicated in several types of cancer including adenomas, breast cancer, lung cancer, ovarian cancer, and HNSCC [65]. *IGF1R* is amplified in 4% of HNSCC tumors from TCGA, placing it among the most commonly amplified genes in this tumor type [13–15]. Both IGF-1R and IGF binding protein-3 (IGFBP-3) have overt impact on HNSCC prognoses, as clinical data reveal that high expression of IGFBP-3 as well as the co-expression of IGFBP-3 with IGF-1R may predict poor prognosis in this cancer type [66]. Preclinical models also demonstrate the role of the IGF-1R signaling pathway in HNSCC: small molecule IGF-1R inhibitor BMS-536924 is effective in both cell lines derived from transgenic mice that spontaneously developed salivary gland tumors and in xenograft mouse models [67].

The tyrosine kinase activity of IGF-1R suggests that the activation of IGF-1R could induce activation of PI3K signaling [68, 69]. Interestingly, studies suggest that the activation of mTOR signaling could be part of a negative feedback loop to reduce PI3K signaling by the phosphorylation of insulin receptor substrate-1 (IRS-1) [70]. This complex relationship between the PI3K and IGFR signaling pathways indicates that IGFR signaling could be a compensatory mechanism contributing to PI3K inhibitor resistance, a phenomenon that has been previously observed in multiple other cancer types [71, 72]. Recent data (submitted) also indicate the potential efficacy of PI3K and IGF-1R inhibitor combinations in HNSCC models, but such dual-therapies have yet to be evaluated in patients.

3.5 Anaplastic lymphoma kinase (ALK)

Aberration in the *ALK* gene is associated with several cancer types; gene fusions have been noted in anaplastic large cell lymphoma and one subset of non-small cell lung cancer [73], while mutations are present in nearly 15% of neuroblastomas [74]. ALK has also been shown to have a pleiotropic role in the aggressive growth and invasiveness of oral squamous cell carcinoma [75]. As a downstream member of the ALK signaling pathway, PI3K/AKT signaling may be activated followed by ALK activation [76]. Collectively, these data motivate consideration of ALK signaling as a compensatory signaling pathway responsible for resistance to PI3K inhibitor monotherapies.

ALK signaling has not historically been evaluated as a resistance mechanism in HNSCC, since fusion events are rare and ALK expression is often quite low in this cancer type. Nevertheless, recent work has shown that EGFR inhibitor treatments may increase the expression of ALK and display greater efficacy with ALK inhibitors [77, 78]. Gonzales et al. showed that the combination of ALK inhibitor and EGFR inhibitor could decrease HNSCC cell proliferation in vitro as well as reduce the volumes of xenotransplantation tumors in vivo [77]. These studies demonstrate that ALK signaling is indeed an important mediator of drug resistance in some head and neck cancers. Additionally, *ALK* was also identified in a CRISPR/Cas9 knockout screen as one of the kinases responsible for resistance to PI3K inhibitor treatment (submitted). PI3K and ALK dual-therapy, however, has not been considered in HNSCC. The only previous studies of PI3K and ALK inhibitors examine such combinations in the context of other tumor types driven by ALK signaling due to fusion with *EML4* or other genes [79–82]. Our recent work shows that co-treatment with ALK inhibitor brigatinib and PI3K inhibitor pictilisib is synergistic in HNSCC models (submitted); this may represent the first evidence of interaction between PI3K and ALK signaling in the absence of *ALK* gene fusion.

However, brigatinib is not a perfectly selective inhibitor of ALK—it also displays activity at IGF-1R, EGFR, and other RTKs, especially at higher concentrations [83, 84]. While ALK inhibition may be an important component of the response to combinations of brigatinib and PI3K inhibitor, we cannot exclude the possibility that the blockade of IGF-1R and/or EGFR contribute at least partially to this response. Both small molecule profiling studies and subsequent validation analyses show that IGF-1R or EGFR inhibition can improve responses to PI3K-targeting therapy, as noted above. Genetic knockouts of *ALK* and *IGF1R* that were generated in combination-responsive HSC-4 cells offer insight into the effect of blocking an individual RTK, mimicking a perfectly selective pharmacologic treatment. *ALK* knockout HSC-4 cells are more sensitive to monotherapy with AKT inhibitor GDC-0068 than wildtype HSC-4 cells. Responses to PI3K inhibition are not markedly different in the knockout model, suggesting that ALK alone may not be responsible for the synergy of ALK- and PI3K-targeting agents in HNSCC. In spite of this, *ALK* and *IGF1R* knockout cell lines display increased levels of p110alpha, the protein encoded by the *PIK3CA* gene (submitted). This indicates an important molecular relationship between the ALK, IGF-1R, and PI3K pathways and may explain the lack of response to PI3K inhibition in knockout cell lines.

While the mechanistic basis for responses to pictilisib and brigatinib has not been fully elucidated, co-treatment with these agents three times per week inhibited tumor growth in a cell line-derived xenograft mouse model (submitted). However, despite the significant result observed after a 3-week course with these inhibitors, tumors did progress as treatment continued for extended lengths of time. This observation is in support of the development of additional compensatory mechanisms mediating treatment resistance. Although the combination of PI3K and ALK inhibitor extended survival for weeks past what would have been observed in mice with vehicle- and brigatinib-treated tumors, further exploration of compensatory signaling in HNSCC and the development of improved treatment paradigms is needed.

3.6 Fibroblast growth factor receptor (FGFR)

FGFRs also have been nominated as a critical candidate for compensatory signaling pathway in HNSCC. After the binding of the fibroblast growth factor (FGF) ligands, the FGFR signaling pathway will modify downstream phosphorylation and gene expression. Importantly, this pathway also has a well-described role in HNSCC pathogenesis due to recurrent of genetic alterations that occur in the disease [85]. According to previous studies, recurrent FGFR1 amplification is present in 17% of OSCC cases [86]. A high level of FGFR2 expression, FGFR3 expression and FGFR2/FGFR3 co-expression are also observed in HNSCC cell lines [87], and such alterations have been suggested to contribute to the early stages of tumor initiation and disease progression [88]. In fact, previous in vitro and in vivo studies show that inhibition of the FGFR signaling pathway could decrease the proliferation of HNSCC supporting cells (fibroblast and endothelial cells) and ultimately results in a decline of tumor cell proliferation through increased cell apoptosis [89]. Combinations of EGFR and FGFR inhibitors have been evaluated in both HNSCC and other cancers, most notably lung cancer [90, 91], but have not translated to the clinic due to toxic effects in patients [92]. Based on these and other data demonstrating the important role of FGFR signaling in HNSCC [93], combinations of PI3K and FGFR inhibitors might be synergistic in a subset of patients.

3.7 Focal adhesion kinase (FAK)

Although most pathways nominated as drivers of PI3K inhibitor resistance are RTKs, a potential relationship between PI3K and the cytoplasmic FAK receptor has also been described. FAK, which is encoded by the Protein Tyrosine Kinase 2 (*PTK2*) gene [94], has a wide range of intracellular functions, involving regulation of cell adhesion, cell proliferation, migration, and cell apoptosis, all of which are commonly altered in head and neck cancer [95]. One previous study assessed 147 HNSCC clinical tumor samples and reported FAK protein overexpression and *PTK2* gene amplification in 62 and 39% of these samples, respectively, supporting a molecular role for this kinase in HNSCC [96]. FAK inhibitors serve to arrest the cell cycle and decrease tumor cell viability via the induction of apoptosis [97]. One such inhibitor, TAE226, is able to induce the tumor cell apoptosis and in turn suppress HNSCC growth in vitro; this inhibitor also blocked tumor growth, metastasis, and angiogenesis in vivo [98]. The mechanisms by which the FAK signaling pathway is involved in tumor cell metastases and growth may be kinase-dependent; in this case, FAK is upstream of PI3K signaling pathway. One hypothesis is that activation of FAK could induce the PI3K and subsequent downstream signaling including remodeling of the cytoskeletal, matrix metalloproteinases (MMPs) as well as formation or turnover of focal adhesion. Such effects might contribute to the metastases of tumor cells. Activation of the FAK-PI3K signaling pathway has also been shown to inhibit tumor cell apoptosis [99–102]. Interestingly, loss of *PTEN*, has been shown to stimulate FAK and downstream targets [103]. However, a role for FAK inhibition has not been well described in cases of *PIK3CA* amplification or mutation. One clinical trial (NCT02372227) sought to evaluate the safety of combining PI3K and FAK inhibitors VS-5584 and VS-6063 in malignant mesothelioma but was terminated prior to completion. A recent report (submitted) shows that FAK/IGF-1R inhibitor TAE226 may be synergistic with PI3K inhibitors in HNSCC cell lines and xenografts, but the contribution of FAK signaling and the mechanism for this effect have yet to be fully determined. Taken together, however, this collection of data nominates FAK signaling as a means of resistance to PI3K inhibitor monotherapy in HNSCC.

3.8 Aurora kinase A (AURKA)

Aurora kinase A (AURKA) is another player identified as having a potentially indispensable role in PI3K inhibitor response in some HNSCCs. AURKA is one of the aurora serine-threonine kinase family members, which play an important role in the regulation of cell cycle and cell division by controlling mitosis and meiosis [104]. In normal cells, AURKA regulates mitosis by contributing to maturation of the centrosome, synthesizing of bipolar spindle, and controlling cytokinesis. Researchers also showed that the upregulation of AURKA is associated with worse prognosis and decreased survival of patients with HNSCCs [105–107]. These poor outcomes are postulated to result from associated centrosome abnormalities and chromosomal aneusomy as well as activation of the spindle assembly checkpoint [106, 108]. AURKA is also involved in both AKT and FAK signaling pathways, and this AURKA/AKT/FAK signaling axis can be responsible for the migration and invasion of HNSCCs [109]. Previous research shows that suppression of AURKA via shRNA could inhibit the ability of laryngeal carcinoma cells to grow and invade both in vitro and in vivo, suggesting that AURKA inhibitor monotherapy may have a substantial effect on oncogenic phenotypes in some models [110]. Furthermore, recent data (submitted) indicate that the regulation of cell cycle gene expression and protein levels, including AURKA, is reduced by synergistic PI3K inhibitor dual-therapies. The altered function of AURKA shows that aurora kinases could play a pivotal compensatory signaling role in PI3K inhibitor resistance.

3.9 Cell apoptosis

Overactivation of PI3K/AKT pathway can inhibit tumor cell apoptosis and promote cell proliferation. The family of forkhead transcription factors (FOXOs) is among the specific downstream PI3K/AKT effectors involved in the regulation of cell apoptosis. The PI3K/AKT pathway mediates serine/threonine phosphorylation of FOXO transcription factors, which reverses the pro-apoptotic effect of FOXOs by downregulating the pro-apoptotic protein BIM, a Bcl-2 family member. Conversely, the use of PI3K and AKT inhibitors may induce cell apoptosis in various tumor types, a phenomenon already detailed in breast cancer models [111]. PI3K/AKT inhibition reduces FOXO phosphorylation leading to the upregulation of BIM [112], an effect that may contribute to apoptosis. The role of specific FOXO family members and their transcriptional targets as well as how these functions are altered by modulation of PI3K signaling has yet to be fully determined in HNSCC.

3.10 PDK1, AKT, and mTOR

Much of the previous work on compensatory resistance to PI3K inhibition has noted contributions from pathways downstream of PI3K or other codependent RTKs, including PDK1 [30], AKT [113], mTOR [114–116], and MEK [117, 118]. Previous publications [117, 118] show that inhibiting PI3K and MEK is functionally similar to inhibiting PI3K and EGFR (see above). Prior studies have also highlighted the importance of PI3K effector AKT in combination responses; these publications describe similar evidence of synergy when replacing PI3K inhibitors with AKT inhibition or siRNA [46] and report reduced AKT phosphorylation following combination PI3K and EGFR inhibitor treatments [44, 46, 47, 82, 119]. Similarly, the work of Sambandam et al. demonstrates the importance of PDK1 inhibition in PI3K inhibitor responses by showing that: (1) reductions in the level of phosphorylated and total PDK1 were present in cell lines that were more sensitive to PI3K inhibition and (2) AKT inhibitor MK-2206 and PDK1 inhibitor GSK2334470 were synergistic when used together, recapitulating or exceeding the effects of PI3K inhibitor monotherapy [30]. In contrast, other analyses (submitted) demonstrate that AKT phosphorylation was similarly reduced following treatment with PI3K monotherapy and ineffective combinations as compared to treatment with synergistic drug pairs. This may suggest that AKT inhibition is necessary, but not sufficient, for response to PI3K inhibitor therapy. PDK1 phosphorylation was also unchanged with PI3K mono- and dual-therapy in this recent dataset (submitted), and little benefit was noted with PDK1, AKT, or mTOR inhibitors to PI3K inhibitors. Based on these findings, it seems that although PDK1, AKT, and mTOR are downstream effectors common to PI3K and the other RTKs involved in synergistic drug pairs, additional mechanisms are responsible for combination effects.

Clinical data evaluating the contribution of targeting both PI3K and its downstream PI3K remain immature. Trials have been performed to determine the safe dose of PI3K inhibitor BEZ235 in combination with mTOR inhibitors RAD001 (NCT01482156) or everolimus (NCT01508104), but results have yet to be published. While there is a trial of PI3K/mTOR inhibitor bimiralisib (NCT03740100) currently ongoing, the contribution of PI3K and mTOR individually will be impossible to ascertain from this study alone.

3.11 Immunotherapy

Emerging data suggest that the PI3K pathway may interact with immune responses and improve the efficacy of immune checkpoint receptor (ICR) blockade. One indication of the involvement of the PI3K pathway in responses to immunotherapies is based on data using models with loss of tumor suppressor *PTEN*. In multiple cancer types, increased expression of programmed death ligand-1 (PD-L1) has been observed in models with loss of *PTEN* [120–123], and there is some evidence that this may also be the case in HNSCC [123].

PI3K signaling also may interface with immune responses through its important role in cellular metabolism. In cancerous cells, the metabolic balance shifts from oxidative phosphorylation to aerobic glycolysis as part of a paradigm known as the Warburg effect [17]. A notable consequence of this effect is the inefficient use of glucose, which limits the maximum possible amount of ATP or energy. Since PI3K pathway activity contributes to glucose uptake and glycolysis [125], cancer cells displaying PI3K pathway activation (through *PIK3CA* mutation, *PTEN* loss or other genetic or functional changes) may utilize additional glucose from the tumor microenvironment. Nearby cytotoxic T-lymphocytes (CTLs) and other immunomodulatory cells, which require vast amounts of glucose to launch and maintain effective immune attack on tumor spread, are therefore in heated competition with PI3K pathway-activated cells for the limited glucose supply. As such, CTL activation, including migration to the tumor site, production of cytokines, and other immune functions, is limited and CTL exhaustion occurs more rapidly [126–130]. PI3K inhibition, however, alters cellular metabolism to prevent or delay CTL exhaustion and thereby allows for a faster and/or more durable CTL response.

Additionally, PI3K inhibition may enhance the effects of ICR blockade by preventing adaptive resistance. One mechanism that may blunt responses to ICR blockade involves the compensatory upregulation of other ICRs. Shayan and coworkers showed that in HNSCC tumor samples, two ICRs, programmed cell death receptor-1 (PD-1) and T-cell Ig and mucin domain-3 protein (TIM-3), were co-expressed on CTLs displaying high levels of exhaustion [131]. PD-1 blockade resulted in further upregulation of TIM-3; this upregulation was reversible using PI3K inhibition [131]. Thus, through multiple means, PI3K inhibition may augment responses to immunotherapy in HNSCC. Clinical trials to evaluate therapies that combine PI3K inhibitor and ICR blockade are ongoing (including NCT04317105 to evaluate PD-1 inhibitor nivolumab and PI3K inhibitor copanlisib in solid tumors), and the results of these studies will offer critical insights for the future of targeted and immunotherapies in HNSCC and other cancers.

4. Conclusions

Due to advances in diverse profiling strategies ranging from next generation sequencing to combinatorial high throughput small molecule profiling to pooled CRISPR and shRNA screens, recent technological advances have led to significant scientific advances in understanding the mechanisms that drive response to PI3K inhibitors in HNSCC and other cancers. Indeed, the future is bright for the advancement of PI3K inhibitors, especially as combination therapies, in HNSCC and other cancer types. Nevertheless, multiple questions remain regarding the role of specific signaling pathways in PI3K inhibitor resistance, and additional studies will be required to further our understanding of this important intersection of pharmacology and cancer biology. More work is needed to develop safer, more effective drugs, to establish biomarkers for response, and to target critical resistance mechanisms. The studies described in this chapter serve as a contribution in these efforts. With the combined efforts of the community, PI3K inhibitors may, in time, have a place among standard-of-care treatments for HNSCC.

Conflict of interest

The authors declare no conflict of interest.

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