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The determinants of head and neck cancer: Unmasking the PI3K pathway mutations

Fernanda S. Giudice[1,2] and **Cristiane H. Squarize**[1,*]

[1]Laboratory of Epithelial Biology, Department of Periodontics and Oral Medicine, School of Dentistry, University of Michigan, Ann Arbor, Michigan, 48109-1078, USA

[2]International Research Center, A. C. Camargo Cancer Center, São Paulo, SP, Brazil

Abstract

Studies attempting to identify and understand the function of mutated genes and deregulated molecular pathways in cancer have been ongoing for many years. The PI3K-PTEN-mTOR signaling pathway is one of the most frequently deregulated pathways in cancer. *PIK3CA* mutations are found 11%–33% of head and neck cancer (HNC). The hotspot mutation sites for *PIK3CA* are E542K, E545K and H1047R/L. The PTEN somatic mutations are in 9–23% of HNC, and they frequently cluster in the phosphatase domain of PTEN protein. *PTEN* loss of heterozygosity (LOH) ranges from 41%–71% and loss of PTEN protein expression occurs in 31.2% of the HNC samples. PIK3CA and PTEN are key molecules in the PI3K-PTEN-mTOR signaling pathway. In this review, we provided a comprehensive overview of mutations in the PI3K-PTEN-mTOR molecular circuitry in HNC, including PI3K family members, TSC1/TSC2, PTEN, AKT, and mTORC1 and mTORC2 complexes. We discussed how these genetic alterations may affect protein structure and function. We also highlight the latest discoveries in protein kinase and tumor suppressor families, emphasizing how mutations in these families interfere with PI3K signaling. A better understanding of the mechanisms underlying cancer formation, progression and resistance to therapy will inform selection of novel genomic-based personalized therapies for head and neck cancer patients.

Keywords

review; tumor sequencing; HNSCC; oncogenomics; mutations

Introduction

Head and neck squamous cell carcinoma (HNSCC) represents 90% of all malignant tumors in the head and neck region. It is formed by a solid mass of epithelial cells in various anatomical sites, such as oral mucosa, tongue, oropharynx, hypopharynx, and larynx. Although HNSCC has very standard histological features, the anatomical sites differ, and various risk factors (e.g., tobacco and HPV infection) contribute to its clinical behavior and

[*]**Corresponding author**: Cristiane H. Squarize, Laboratory of Epithelial Biology, Department of Periodontics and Oral Medicine, Division of Oral Pathology and Radiology, School of Dentistry, University of Michigan, 1011 N. University Ave., Room 3210, Ann Arbor, MI, USA - 48109-1078, Phone: (734) 615-3406, csquariz@umich.edu.

response to therapy. This tumor is remarkably heterogeneous and success of future treatments relies on a better understanding of its diversity; this will allow us to identify groups of patients with similar HNSCC biological behavior and response to therapy. A major challenge in the scientific community is assessing the heterogeneity of HNSCC.

Large screening projects, such as the Head and Neck Genome Anatomy Project (HN-CGAP), started more than 10 years ago (http://cgap.nci.nih.gov) and were followed by the International Cancer Genome Consortium (ICGC) [1]. These joint efforts led to whole-exome sequencing analysis of HNSCC, resulting in identification of the most common genomic alterations [2, 3]. These results confirmed that HNSCC is extremely heterogeneous and there is no single genetic alteration or dysregulated molecular pathway responsible for its development and progression. Rather, it is evident that distinct genetic tumor signatures and dysregulated signaling pathways are shared within HNSCC subtypes, which may result in different clinical courses of the disease. In order to further advance therapy and improve survival of HNSCC patients, it is likely that patients with varying HNSCC should not be treated similarly.

Based on dysregulated pathways and genetic signatures in HNSCC, new therapies need to be developed that target specific subgroups of patients. For example, HPV-associated HNSCC became a subgroup of HNSCC with a distinct prognosis and response to therapy [4]. HPV-positive HNSCC displays several mutations but less than HPV-negative HNSCC. Indeed, the mutation rate in HPV-positive tumors is approximately half the mutation rate in HPV-negative HNSCC, suggesting that HPV oncogenes may play a role with acquired mutations to promote HPV-associated HNSCC [2]. Furthermore, additional studies revealed that *PIK3CA* or *PIK3R1* were the only mutated genes in a subset of HPV-positive HNSCC [5].

The overall mutation rate of HNSCC is similar to other smoking-related tumors, such as small cell lung cancer and lung adenocarcinoma [2, 6]. HPV-negative HNSCC has a great diversity of genetic alterations in a variety of critical genes, which may underlie HNSCC formation, progression, and response to therapy. Mutations in the *TP53* gene are common and occur in little over 50% of HNSCC, and mutations in *Notch* genes (*NOTCH1, NOTCH2, and NOTCH3*) are present in ~22% of HNSCC. Remarkably, PI3K/mTOR pathway genes, such as *PTEN, TSC1* and *PIK3CA*, comprise 30.5% of the mutations found in HNSCC [3, 6]. Regardless of genomic heterogeneity, the phosphoinositide 3-kinase (PI3K) pathway is the most frequently somatically mutated pathway in HNSCC [5].

As seen in Figure 1, the PI3K pathway impacts most cellular functions involved in tumor behavior, including cell growth, local invasion, metastasis, survival, and resistance to therapy [7–9]. Genomic aberrations in HNSCC include posttranslational alterations and mutations in proteins downstream of PI3K and PTEN, including AKT, TSC1/TSC2, mTOR, RICTOR, and RAPTOR [2, 3, 5, 10]. Additional gene mutations and amplifications contribute to aberrant activation of the PI3K pathway. For example, increased Receptor Tyrosine Kinase (RTK) (e.g., EGFR, ErbBs, and MET) signaling induces PI3K/PTEN pathway activation [11]. Mutations in Ras genes (i.e., HRAS and KRAS) activate the PI3K

pathway via p110α [12–15] and loss of p53 function promotes mTORC1 activation and regulation of PTEN transcription [16–18].

This article reviews the profiling of genetic alterations in the PI3K/PTEN/mTOR pathway in HNSCC to understand how mutations alter HNSCC behavior and phenotype. This is an essential step towards personalized treatments of HNSCC using genomic-based therapy selection.

PTEN mutations

Phosphatase and Tensin homologue (PTEN; also known as MMAC1 and TEP1) is frequently mutated in multiple cancers [19, 20]. PTEN is well-known for its tumor suppressor role as a dual protein phosphatase. PTEN dephosphorylates phosphatidylinositol 3,4,5-trisphosphate (referred to as PIP3, *PtdIns*(3,4,5)*P*, or PI(3,4,5)P3) into phosphatidylinositol 4,5-bisphosphate (referred to as PIP2, *PtdIns*(4,5)*P*, or PI(4,5)P2), thereby broadly inhibiting oncogenic PI3K signaling. AKT relies on PIP3 for activation, an effect that is opposed by PTEN. Consequently, loss of PTEN function leads to increased oncogenic activity and enhanced PIP3 levels and PI3K/mTOR signaling. The phosphatase domain is the functional area of the PTEN protein responsible for modulating the levels of PIP3 (Figure 1A)[9].

PTEN encodes for a protein with 403 amino acids and 2 major functional domains, the N-terminus and the C-terminus domains. Exons 1–5 encode the N-terminus, and exons 6–9 encode the C-terminus. Particularly, exon 5 encodes for the phosphatase domain [21] (Figure 2A).

Point mutations in the *PTEN* gene on 10q23.3 locus have been found in 9–23% of HNSCC (Table 1) [2, 3, 22–24]. Although not well understood, *PTEN* synonymous or silent mutations in HNSCC were identified in introns (i.e.,1, 2, 4, 6 and 7) and in exon 1 prior to the PTEN start codon (Figure 2A). To date, nonsynonymous mutations in *PTEN* have been found in exons 5–8. Recurrent mutations occur primarily in exon 5 codons, which results in p. D92E, p.D92G, and p. A121E at the phosphatase domain. In addition, recurrent mutations were also found in exon 8, resulting in p.D331G at the C2 domain (Figure 2A). Although the mutations appear dispersed mostly on exons 5–8 of the *PTEN* gene, the resulting amino acid changes are non-randomly distributed. Notably, the amino acids changes are concentrated in the functional areas of the PTEN protein. As seen in Figure 2B, nonsynonymous mutations in HNSCC cluster in the phosphatase domain, disrupting the functional area between active site cysteine residues 71 (C71) and 124 (C124). PTEN mutation in residue R14 has been described in HNSCC (Table 1). R14 mutation participates in PTEN decrease nuclear localization and it is important for PTEN membrane biding and catalytic activity [25, 26].

Sporadic somatic mutations in *PTEN* are not an isolated event in HNSCC. Rather, they are one of the most common genetic alterations in human malignancies, particularly in solid tumors [19, 20]. However, the functional and biological roles of these mutations in HNSCC are largely unknown. Insights into PTEN structure and its role in various biological processes may help us understand how *PTEN* mutations and loss of expression contribute to HNSCC development, progression, and response to therapy.

The crystal structure of PTEN reveals important details about its three-dimensional folding that can add to our understanding of potential associations between distinct mutations and functional changes in HNSCC. The structure of the phosphatase domain displays an active pocket rich in basic residues that are essential for the ability of PTEN to dephosphorylate its substrates (i.e., acid phospholipids). The active pocket is also wide enough to fit the phosphorylated inositol moiety of PIP3. This active site has 3 catalytic loops: WPD-loop (residues 88–98), P-loop (residues 121–131) and TI loop (residues 160–171). Mutations in these phosphatase-binding loops are frequently found in human cancer, patients with hereditary PTEN related syndromes, and neurodevelopmental disorders, such as autism spectrum disorders (ASDs) [21, 27]. PTEN mutations in the phosphatase-binding loops in HNSCC are restricted to the WPD-loop (p.Y88C, p.F90L, p.D92G, p.D92E) and the P-loop (p.A121E) (Figure 3A and 3B). The PTEN catalytic signature (HCKAGKGR) is comprised of residues 123 to 130 and forms the bottom of the active pocket. The wall of the pocket is partly formed by the WPD-loop and the TI-loop [21, 27].

The amino acids within the phosphatase domain are a common area for cancer-associated point mutations to arise, and they can lead to a reduction or abrogation of PTEN phosphatase activity. In order to understand the effects of PTEN mutations, extensive site-direct mutagenesis using comprehensive Ala–scanning mutagenesis (Ala-substitutions) was performed in the phosphatase binding loops of PTEN [27]. A humanized-yeast assay revealed that mutations in ASD influence the catalytic properties of PTEN. The Ala-substitutions in the WPD-loop resulted in partial enzymatic activity of PTEN. Interestingly, the p.D92E, p.D92G, and p.A121E mutations resulted in the inability of PTEN to catalyze PIP3 and P110α (catalytic subunit of class I PI3K) mediated growth. Conversely, p.Y88C and p.F90L did not alter PTEN lipid phosphatase activity, although p.Y88C partially affected growth in yeast [27]. Certain mutations, including p.Y88C, p.92AG, p.Y225C and a silent mutation (281A>T), were also paired with loss of heterozygosity (LOH) of chromosome 10 that involves the PTEN locus [23].

Located at the carboxy-terminus (C-terminus) is the C2 domain, a region important in protein-protein interaction, lipid membrane binding, and PTEN protein stability. The C2 domain (residues 192–350) binds to phospholipids at the cell membrane and allows PTEN to modulate cell migration. In HNSCC, p.M205I, p.Y225C, p.Q245*, p.P246L, p. D252Y, p.D331G, and p. R335* mutations are found in the C2 domain (Figure 3C and Table 1). There is little information regarding how mutations in the C2 domain affect PTEN function. The p.P246L and p.R335L mutations have also been identified in PTEN hereditary syndromes. Although located in the C2 domain, these two mutations partially interfere with PTEN lipid phosphatase activity and cell growth [27]. This may be related to the ability of the C2 domain to position the PTEN phosphatase active site towards to the cell membrane. Expression of the p.D331G mutation, located in the C2α loop, significantly reduces PTEN expression in U-87 MG glioblastoma cell lines. These results are consistent with the role of the C2 domain in PTEN protein stability [28].

C2 domain mutations found in HNSCC are closely related to mutations in endometrial carcinoma (D331G and S227F), lung cancer (G251C), and glioblastoma (P204S) [29–32]. P204S (present in the Cβ1/2 loop) and G125C mutations are associated with reduced PTEN

expression and the inability to suppress cell growth [28]. Mutations in the C2α loop (i.e. D331G) and the Cβ1/2 loop (i.e. pM205I) likely affect PTEN protein stability and catalytic activity. Nonsense (i.e., p.Q245* and p.R335*) mutations, frameshift mutations, and mutations that produce splice variants also occur in the C2 domain and can prematurely terminate the open reading frame of *PTEN* to produce a truncated and nonfunctional protein. These newly described HNSCC PTEN mutations are located in important functional areas of PTEN and their effects on protein function and biological implications in HNSCC are unknown.

In addition to mutations, loss of heterozygosity (LOH) on chromosome 10 in the *PTEN* gene locus region is detected in HNSCC tissue samples at frequencies ranging from 41% to 71% [18, 22, 23, 33]. A second inactivation event (mutation or homozygous deletion) among the LOH of *PTEN* is only detected in up to 25% of HNSCC, suggesting that a single functional copy of *PTEN* in epithelial cells is insufficient for PTEN to function as a tumor suppressor (i.e.,*PTEN* haploinsufficiency). Additionally, LOH on chromosome 10 in the PTEN locus and loss of PTEN expression correlate to invasive and poorly differentiated carcinomas, lymph node involvement/metastases, and poor prognosis of HNSCC. Furthermore, loss of PTEN protein expression occurs in 31.2% (221/709) of human HNSCC samples [34].

Evidence from animal models implicates PTEN in epithelial lesions and benign tumors [35, 36]. To understand the biological relevance of PTEN alterations (i.e., mutations, LOH, and complete loss of function) and PI3K/mTOR activation in HNSCC initiation and progression, a novel oral specific HNSCC animal model was developed [34]. This genetically and environmentally defined HNSCC animal model has *Pten* LOH and full deletion of PTEN in the intraoral epithelia. Conditional deletion of *Pten* was achieved using the keratin promoter 14 CRE-lox system. Because tobacco is the most common etiological factor in HNSCC, animals were exposed to a tobacco surrogate (4-nitroquinoline-1-oxide or 4NQO) to increase their susceptibility to oral cancer. Deactivation and/or downregulation of PTEN due to mutations, epigenetic changes, or posttranslational regulation were recapitulated by hemizygous (partial) or homozygous (complete) deletion of the *Pten* gene. *Pten* ablation also resulted in increased activation of PI3K and other pathways. These mice showed rapid development of oral-specific premalignant lesions and carcinomas [34]. Notably, HNSCC with deregulated PTEN lack p53 and show overexpression of pAKT and mTOR. In addition, animals lacking PTEN expression have increased COX2 expression and angiogenesis in the surrounding stroma [34].

PI3K family gene mutations

PI3Ks belong to a family of intracellular lipid kinases that phosphorylate the 3′-hydroxyl group of phosphatidylinositol (also known as PI or *PtdIns*) and phosphoinositides. The lipid products of PI3K act as second messengers by binding and activating several signaling pathway molecules.

PI3K signaling is involved in the control of fundamental cellular functions and properties, including cell growth, proliferation, differentiation, survival, metabolism, vesicular trafficking, degranulation, cytoskeletal rearrangement, and motility (Figure 1) [8, 9, 37].

PI3Ks are divided into Class I, Class II, and Class III according to their structural features, *in vitro* lipid substrate specificity, tissue distribution, mechanism of activation, and function (Figure 6) [8, 12].

The *in vitro* substrates for class I PI3Ks are PI, Phosphatidylinositol 4-phosphate (referred to as PI(4)P or *PtdIns4P*), and PIP2; and the primary *in vivo* substrate is PIP2. Therefore, class I PI3Ks primarily catalyzes the conversion of PIP2 to PIP3. PIP3 activates many downstream signaling proteins, including the protein serine-threonine kinase AKT. The lipid phosphatase PTEN dephosphorylates PIP3, thereby regulating PI3K signaling [12].

Class I PI3K isoforms are comprised of p110α (also called PIK3CA), p110β (also called PIK3CB), p110γ (also called PIK3CG), and p110δ (also called PIK3CD). In mammals, Class I PI3Ks is divided into two groups, Class IA and Class IB, based on structural and functional differences. Receptor tyrosine kinases (RTKs), such as epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (HGFR or MET), platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), and insulin-like growth factor 1 receptor (IGF-1R), act as primary activators of Class IA PI3Ks. Hras also activates Class I PI3Ks isoforms [13, 14]. Hras is frequently mutated in HNSCC, specially in Asia [14], and its interaction with p110α is mediated by the RBD domain in p110α. The Class IA PI3Ks isoform, PIK3CA, is a critical effector for RAS-driven tumorigenesis [15]. Additionally, G-protein-coupled receptors (GPCRs) activate Class IB PI3Ks (GPCRs) [12, 37, 38].

Class IA is the most common PI3K class involved in human cancers [12]. Class IA PI3Ks are comprised of p110 catalytic subunits (i.e., p110α, p110β, and p110δ) that form heterodimeric protein complexes with p85 regulatory subunits. p110α, p110β, and p110δ are encoded by *PIK3CA*, *PIK3CB*, and *PIK3CD* genes, respectively. There are five isoforms of the p85 regulatory subunit, including p50α, p55α, p55γ, p85α, and p85β.p85α, and its splices variants p50α, p55α, are encoded by a single *PIK3R1* gene, but p85β and p55γ isoforms are encoded by *PIK3R2* and *PIK3R3*, respectively [12]. Binding of p85 subunits with p110 subunits promotes stabilization of p110 subunits. Once at the plasma membrane, p110 subunits phosphorylate its lipid substrates [12, 37, 39, 40].

Class IB PI3Ks are found exclusively in mammals. They are formed by dimerization between the p110δ and p101 (also called PIK3R5) or p87 (also called PIK3R6, $p87^{PIKAP}$ or p84). p101 is encoded by the *PIK3R5* gene, p87 is encoded by the *PIK3R6* gene, and the p110γ is encoded by the *PIK3CG* gene. GPCRs activate Class IB PI3Ks through a mechanism in which p101 recruits p110γ, permitting their activation and translocation to the plasma membrane by βγ subunits of trimeric G proteins [41–43]. The p110α and p110β are ubiquitously expressed, and p110δ and p110γ are primarily found in the hematopoietic system, especially in leukocytes [44]. This tissue-specific distribution suggests a more specific and essential function of PI3Kδ and PI3Kγ signaling in the microenvironment of tumors and in hematopoietic tumors and diseases.

Class II PI3Ks have three mammalian isoforms named PI3K-C2α (also called PIK3C2A), PI3K-C2β (also called PIK3C2B), and PI3K-C2γ (also called PIK3C2G), which are encoded

by *PIK3C2A*, *PIK3C2B* and *PIK3C2G* genes, respectively [45–49]. PI3K-C2α and PI3K-C2β are ubiquitously expressed, and PI3K-C2γ is a liver-restricted protein [50]. Unlike class I PI3Ks, class II PI3Ks are monomers with only a catalytic domain [47]. Class II PI3Ks have well-recognized *in vitro* substrates, PI and PI(4)P, but the *in vivo* targets are still being identified. PI(4)P is used to generate PI(3,4)P2, and PI is used to generate PI3P [12].

Class III PI3K has a single member, the vacuolar protein-sorting 34 (VPS34, also called PIK3C3) that was first identified in mammals in 1995 [51]. It is highly conserved among yeast, plants, and mammals. The catalytic subunit VPS34 is encoded by the *PIK3C3 gene*. VPS34 forms a complex with serine threonine protein kinase p150 (also called VSP15 or PIK3R4), which is encoded by the *PIK3R4 gene*. The regulatory subunit is essential for activation and recruitment of VPS34 to intracellular membranes where it phosphorylates PI into PI3P, the only substrate of class III PI3Ks [12]. The most recognized function of class III PI3Ks is the regulation of vesicular trafficking in the endosomal/lysosomal system [52–56]. Class III PI3Ks also activate additional mechanisms in mammalian cells, such as endocytosis and phagocytosis. Additionally, VPS34 kinase may also play a role in autophagy and protein synthesis through an mTOR-dependent mechanism. PI3P, which is primarily localized in endosomes, phagosomes and intracellular organelles such as endoplasmic reticulum, can be converted to PI(3,5)P2 by the lipid FYVE finger-containing phosphoinositide kinase (PIKfyve, also called FAB1) [57–60].

Regardless of PI3K class distinctions, genetic alterations in PI3K genes and epigenetic aberrations in PI3K signaling are commonly found in cancer development and progression [61–64]. Multiple tumors, including colon, brain, gastric, breast and lung, were sequenced to determine which PI3Ks were mutated. *PIK3CA* was the only gene in the PI3K family with tumor-specific somatic mutations [64]. Several subsequent studies confirmed the presence of *PIK3CA* mutations in solid tumors [62, 64]. Sequencing of ~150 HNSCC tumor samples revealed mutations in the PI3K pathway in 30.5% of cases, making it the most frequently mutated oncogenic pathway in HNSCC [2, 3, 5]. HNSCC tumors harboring PI3K pathway mutations have greater genomic instability than tumors lacking mutations in PI3K genes. These data are supported by the finding that tumors with PI3K pathway mutations have a larger number of non-synonymous and cancer gene mutations than tumors without PI3K mutations. Additionally, tumors with PI3K mutations show increased frequency of mutations in DNA damage/repair genes [5].

Notably, advanced (i.e., Stage IV) ovarian, colorectal, and pancreatic tumors may exhibit concomitant mutational events in PI3K pathway genes and in other pathways, such as MAPK. These concomitant-activating mutations result in a poor response to chemotherapy [65, 66]. Simultaneous mutations in PI3K and MAPK pathways have not been reported in HNSCC [2, 3, 5, 65, 66], indicating that activation of concomitant signaling in these pathways is due to posttranslational activation and/or crosstalk between pathways in HNSCC. Furthermore, advanced stage HNSCC tumors have mutations in more than one PI3K pathway molecule, as evidenced by mutations in *PI3C2G* and *PTEN*; *PIK3R1* and *mTOR*; *PIK3CA* and *mTOR*; *PIK3CA* and *PTEN*; *PIK3C2G*, *PIK3R5* and *PIK3CA* [5] Therefore, combined aberrations in PI3K pathway molecules may be associated with progression of HNSCC, while mutually exclusive mutations and alterations within this

pathway is related to HNSCC formation and initial progression. Indeed, amplification of *PIK3CA* is detected in oral dysplastic lesions, and amplification associated with mutations was associated to invasive HNSCC [67, 68]. Mutually exclusive mutations of PI3KCA and PTEN have also been observed in breast cancer and gastric carcinomas [69, 70].

More than 30% of human solid tumors, such as gastric, lung, breast, hepatocellular, colorectal, ovarian and glioblastoma carcinomas, exhibit mutations in *PIK3CA* [62, 64]. Moreover, *PIK3CA* is mutated in 11%–33% of HNSCC, making it one of the most commonly mutated genes in this disease [2, 3, 5, 71–73]. *PIK3CA* is located on chromosome 3q26.32 and encodes the p110α catalytic subunit of PI3K. In addition to the activating mutations, *PI3KCA* is also amplified in most human malignancies [62, 64], including HNSCCs [5, 67, 74, 75]. *PIK3CA* has been associated with advanced stage tumors, vascular invasion, and metastasis in HNSCC [67, 72, 74, 75]. Across cancer types, *PIK3CA* alterations impact cancer predisposition, progression, recurrence, metastasis, and prognosis [69, 76–81].

The *PIK3CA* mutations in human tumors are somatic, cancer-specific, heterozygous, and primarily missense [62, 64]. The pattern of *PIK3CA* mutation is normally characterized by more than 75% of mutations grouped in the helical (exon 9) and kinase domains (exon 20) of the gene. These domains are described as hot spots for *PIK3CA* mutations [64]. In HNSCC, hotspot mutation sites for *PIK3CA* mutations are E542K, E545K and H1047R/L [5, 64, 82–84], which comprise ~90% of *PIK3CA* mutations identified in HNSCC (Figure 5A and Table 2). Most commonly, the glutamic amino acid in positions 542 and 545 (i.e., E542 and E545) is substituted with a lysine (K), and histidine1047 (i.e., H1047) is frequently substituted with arginine (R).

PIK3CA mutations are activating mutations that often result in biological behavioral changes to the tumor. At all three hotspot mutation sites, aberrations lead to a gain of enzymatic function [64]. For example, in HNSCC, colorectal, breast and ovarian cancers, the primary functional consequence of *PI3KCA* hotspot mutations is elevated lipid kinase activity that results in constitutive AKT signaling, as revealed by increased phosphorylation of AKT and its downstream targets S6K and 4EBP [5, 73, 83, 85–88]. These hotspot mutations can also transform chicken-embryo fibroblasts, NIH 3T3 cells, and mammary epithelial cells [83, 85, 88]. These mutations cause increased proliferation rates and more severe metastatic phenotypes in HNSCC and breast and colorectal cancers, as evidenced by increased migration and invasion *in vitro* and *in vivo* [73, 86, 89].

Recently, four novels *PIK3CA* mutations, R115L, G363A, C971R, R975S, were reported in HNSCC tumors. Functional assays showed increased HNSCC cell growth when R975S, R115L and H1047R were overexpressed and AKT was phosphorylated at threonine 308 and serine 473 [5]. Xenografts of a HNSCC tumor sample containing the *PIK3CA* mutation (E542K) showed a better response to the BEZ235 dual inhibitor compared to HNSCC tumors expressing wildtype *PIK3CA* [5]. These findings support previous studies that showed tumors with altered and mutated PI3K family genes, including PI3KCA and PTEN, are exquisitely sensitive to PI3K/mTOR inhibitors [65, 90–95]. Therefore PI3K/mTOR inhibitors, used as a single or coadjuvant treatment, may be a novel targeted therapy for

HNSCC treatment, especially HNSCC tumors with mutated and altered PI3K signaling molecules.

Janku et al., 2011 detected *PIK3CA* mutations in 11.5% of patients with different solid tumors, including endometrial, ovarian, colorectal, breast, cervical and HNSCC. Patients with *PIK3CA* mutations show a better response to PI3K/mTOR pathway inhibitors compared to patients without PIK3CA mutations [65]. However, as previously mentioned, malignant tumors expressing simultaneous mutations in MAPK and PI3K pathways have poor responses to PI3K/AKT/mTOR pathway inhibitors [65, 66], suggesting that inhibitors that target PI3K pathway effectors in combination with MEK inhibitors could be an effective molecular targeted therapy to treat HNSCC.

In addition to *PIK3CA* mutations, mutations in *PIK3CB* and *PIK3CD*, genes that encode the catalytic subunits p110β and p110δ, respectively, have been found in HNSCC. p110βharborsa synonymous mutation (p.P422P), and p110δ has missense mutations (p.T423S and p.T458S) (Table 2) [2, 5]. The significance of these mutations in PI3K signaling in HNSCC is unknown. Mutation in the *PIK3CD* gene was also described in neuroblastoma, and its biological function correlates to tumor development and progression [96].

Additional PI3K class I components are also mutated in HNSCC (Table 2). *PIK3R1* has an in frame insertion (c.1914–1916 - TAT>TATAAT) and missense mutations (p.I290V and p.D560H). *PIK3R1* encodes p85α, p55α and p50α [97, 98]. Mutations in *PIK3R1 a*lone represent 7% of HNSCC mutations (3/41). Studies have reported p85α mutations in certain human tumors, including breast [99], colon [99, 100], ovarian [100], glioblastoma [101], and HNSCC [5], but their roles in tumor development and/or behavior are still unknown. Mice lacking *PIK3R1* in the liver (L-Pik3r1KO mice) develop aggressive high-grade hepatocellular carcinomas and lung metastases [102]. p.D560Y induces anchorage-independent growth, increased proliferation and AKT activation *in vitro*. Mice implanted with BaF3 cells containing p85α D560Y develop metastases in the liver and spleen [97]. Potential effects of HNSCC mutations are suggested by where they arise in the p85α (Figure 5B). Mutation at D560 occurs in the p85α iSH2 domain, a protein domain that permits formation of a complex with p110α [103]. The p85 regulatory subunit is required to stabilize and inhibit the catalytic activity of p110 in basal conditions [40, 104]. The *PIK3R1* mutation in colon carcinomas disrupts p85 inhibition of p110, resulting in constitutive activation of the PI3K pathway beyond p110α activation. Therefore, p85α mutants may promote AKT activation, cell survival, and oncogenesis in a p110-dependent manner [97]. Further PI3K activation via the p85αmutation may also regulate PTEN because reduced p85α expression is associated with decreased PTEN expression and activity [102, 105, 106].

Mutations in *PIK3CG* (p110γ), *PIK3R5* (p101) and *PIK3R6* (p84/p87^{PIKAP}) were identified in HNSCCs (Table 2). [5]. These PI3K family members belong to Class IB PI3K, but the consequences of these mutations to HNSCC are still unknown. Notably, p110$\gamma^{-/-}$ mice develop tumors in the colon (colorectal adenocarcinomas) [107] and hematologic defects [108–110]. Although mutations in PIK3R5 and PIK3R6 have been identified in lung adenocarcinoma [111], their effects on tumorigenesis are unknown.

Mutations in *PIK3C2A*, *PIK3C2B* and *PIK3C2G*, members of the Class II PI3Ks, were also identified in HNSCC (Table 2). *PIK3C2B* is associated with prostate cancer risk [112], and surprisingly, *PI3KC2B* may promote resistance of leukemia cells to chemotherapeutics [113].

Lastly, the *PIKfyve* gene that encodes for PIKfyve protein is also mutated in HNSCC (Table 2). PIKFyve regulates key cellular processes, such as membrane trafficking, stress-induced signaling, cytoskeletal dynamics, nuclear transport, gene transcription and cell cycle progression [114]. PIKfyve regulation and functionality depends on stable complex formation between PIKFyve and the antagonistic PI(3,5)P2-specific phosphatase Sac3. Sac3 originated from the FIG 4 gene and orchestrates the coordinated regulation of PI(3,5)P2 synthesis and turnover [115]. In addition to HNSCC mutations, dysfunction and mutations in PIKfyve and Sac3 are also found cancers of the ovary, endometrium, and lung, among others (COSMIC database: http://www.sanger.ac.uk/cosmic) [71].

Additional mutations in the PI3K/PTEN/mTOR pathway (*AKT, mTOR, TSC1, TSC2, RICTOR, and RPTOR*)

AKT2 mutations

AKT2 is mutated at p.Y351C in HNSCC (Table 1) [5]. Although Y351 is a putative phosphorylation site in AKT2 that is conserved among multiple species (Figure 6) (http://www.kinexus.ca), its contribution to AKT2 protein function and effect on cells is not understood. AKT2 overexpression also occurs in HNSCC [116].

AKT gene family members are *AKT1, AKT2, and AKT3*, which are ubiquitously expressed in most tissue types (www.genecards.org). These genes encode AKT1/PKB-α, AKT2/PKB-β, and AKT3/PKB-γ proteins isoforms, which share a high degree of amino acid similarity and are activated by similar pathways in a PI3K-dependent manner [117–119]. The well-known phosphorylation sites of AKT are threonine 308 ($pAKT^{Thr308}$) and serine 473 ($pAKT^{Ser473}$). These AKT phosphorylation sites, along with markers of mTOR activation (e.g. PRAS40), have prognostic value and have been implicated in predicting chemotherapeutic response in lung and breast cancer [94, 120, 121].

In HNSCC, AKT activation occurs in premalignant lesions and late stage carcinomas and is correlated with poor clinical outcome [10, 122–124]. Nonetheless, analyses of total AKT function is revealing specific roles for the isoforms [125]. *In vivo* studies using constitutive AKT1 (myrAKT) overexpression resulted in oral lesions that are blocked by senescence; subsequent ablation of Trp53 permits malignant progression [126]. In breast cancer mouse models, AKT1 participates in ErbB-2 induced tumorigenesis and but not in tumor invasion. Upregulation of AKT2 is involved in migration, invasion, metastases, and treatment resistance in neuroblastoma, breast, and colorectal cancers [125, 127–131]. Furthermore, *in vivo* studies showed that AKT2 directly affects glucose uptake [132]. Upregulation of AKT2 is induced during epithelial mesenchymal transition (EMT) by Twist [127]. Conversely, downregulation of AKT2 induces caspase-dependent apoptosis and suppresses EMT and migration [130, 133–135].

TSC1 and *TSC2* mutations

The tumor suppressor genes *TSC1 and TSC2* are on chromosome 9q34.13 and 16p13.3, respectively. TSC1 encodes Hamartin and TSC2 encodes Tuberin, which form heterodimers [136, 137]. The Hamartin/Tuberin formation is referred to as the TSC1/TSC2 complex, which is located downstream of AKT/PKB. AKT phosphorylates and inactivates TSC2, and inactivation of the TSC1/TSC2 complex leads to enhanced mTOR signaling through activation of mTORC1 [138, 139]. AKT can also signal to mTORC1 in a TSC1/TSC2-independent manner by phosphorylating the PRAS40, amTORC1 inhibitor [140, 141]. Pro-inflammatory cytokines important to HNSCC, such as TNFα, also activate mTOR via IKKβ phosphorylation of TSC1 [142]. In addition, Wnt signaling modulates mTOR via TSC1/TSC2 interaction and GSK3 [143].

Mutations in TSC1/TSC2 tumor suppressor genes that result in inactivation of the complex are commonly found in patients with tuberous sclerosis [144–146]. In HNSCC, unique mutations have been described in TSC1 (p.R245*) and TSC2 (p.S1514*) (Table 1) [2, 3] and DNA methylation and LOH in TSC1/TSC2 also occur [147]. How mutations in TSC1/TSC2 contribute to HNSCC may be revealed by the function of the TSC1/TSC2 complex. For example, the TSC1/TSC2 complex is the main regulator of mTORC1, and inactivation of TSC1/TSC2 promotes mTORC1 activation [148]. TSC2 overexpression inhibits growth of HNSCC cells [149].

Mutations in mTOR, Rictor, Raptor, and *FOX* gene families

The *mTOR, RICTOR, and RPTOR* genes are mutated in HNSCC (Table 1). Specifically, the *mTOR* mutations are p.L2260H and p.R1161Q, the RICTOR mutations are p.E1633*and p.D175H, and the RAPTOR mutation is p.P407L [2, 5]. *RICTOR* is also amplified in 38.7% of HNSCC, some of which also contain *PI3KCA* (45.2%) and *EGFR* (32.3%) amplification [82]. It is unclear how these newly described mutations and amplifications affect HNSCC. *mTOR, RICTOR, and RPTOR* genes encode for mTOR, RICTOR and RAPTOR proteins, respectively. The mTOR is the catalytic subunit of two distinctive molecular complexes known as mTOR complex 1 (mTORC1 and mTOR complex 2 (mTORC2) [150]. mTORC1 is comprised of RAPTOR, PRAS40, mTOR, mLST8, DEPTOR, and TTIL/TEL2 complex proteins. mTORC2 has seven known protein components, including RICTOR, mSIN, PROTOR1/2 mTOR, mLST8, DEPTOR, and TTIL/TEL2. mTORC1 and mTORC2 complexes can sense and respond to a variety of endogenous and exogenous signals, including growth factors, stress, oxygen, and amino acids, among others. mTORC1 is a well-known activator of translation regulators and initiation factors, such as p70/S6 and 4E binding protein 1 (4E-BP1) and Garb10 [151]. mTORC1 activation induces cell growth, cell cycle progression, protein and lipid syntheses, and autophagy [150]. In contrast to mTORC1, activation of mTORC2 seems to be independent of nutrients, but it does respond to growth factors stimuli such as insulin (citation). mTORC2 controls several molecules, including AKT, SGK1 (serum/glucocorticoid regulated kinase 1), and PKC-α (protein kinase C-α) [152–154]. mTORC2 phosphorylates AKT at Ser473 [155]. Notably, depletion of mTORC2 impairs AKT signaling, resulting in deficient activation of Forkhead Box proteins, such as FOXO1 and FOXO3A, but having no effect on TSC2 and GSK3 phosphorylation [152–154]. Finally, mTORC2 also controls paxillin and Rho GTPases via

PKC-α [150]. Therefore, mTORC2 regulates cell shape, migration, cytoskeleton organization, survival, and metabolism [150]. *FOX* family genes, including *FOXO1, FOXO3, FOXD1, FOXD3, FOXJ2, FOXK1, FOXI1, FOXN1, FOXN3, FOXP1, FOXP2, and FOXP4*, are also mutated in HNSCC [2, 3]. FOX proteins belong to a large family of transcriptional regulators that affect the behavior of breast, prostate and colon tumors and may serve as a target for therapeutic intervention [61, 156, 157].

In Conclusion

There has been much progress in identifying mutated genes and proteins in HNSCC, but numerous questions remain regarding their physiological and pathological roles. In vitro and in vivo studies using genetic and pharmacological tools are necessary to better understand HNSCC tumor biology and to improve cancer therapy in order to increase patient survival, quality of life, and ultimately, disease resolution. Cancer, particularly of the head and neck, is a very complex genetic disease. The acquisition of oncogenic signals, such those driven by HPV infection and somatic mutations, affect cellular transformation, tumor development, progression, and metastases. Interestingly, distinct cell types, including cancer stem cell, may respond differently to mutations in p53, PI3KCA, PTEN and other proteins. Furthermore, not every group of head and neck cancer patients with distinctive group of mutations or deregulated signaling pathways will respond the same to therapy. Individualized therapies catering these different subgroups of HNSCC tumors may yield better outcomes. Much work is still needed to understand the contribution of these mutations and deregulated pathways to HNSCC and to determine the effects of inhibitors that target molecules in this pathway. This information will guide the discovery of novel drugs and treatment approaches for HNSCC.

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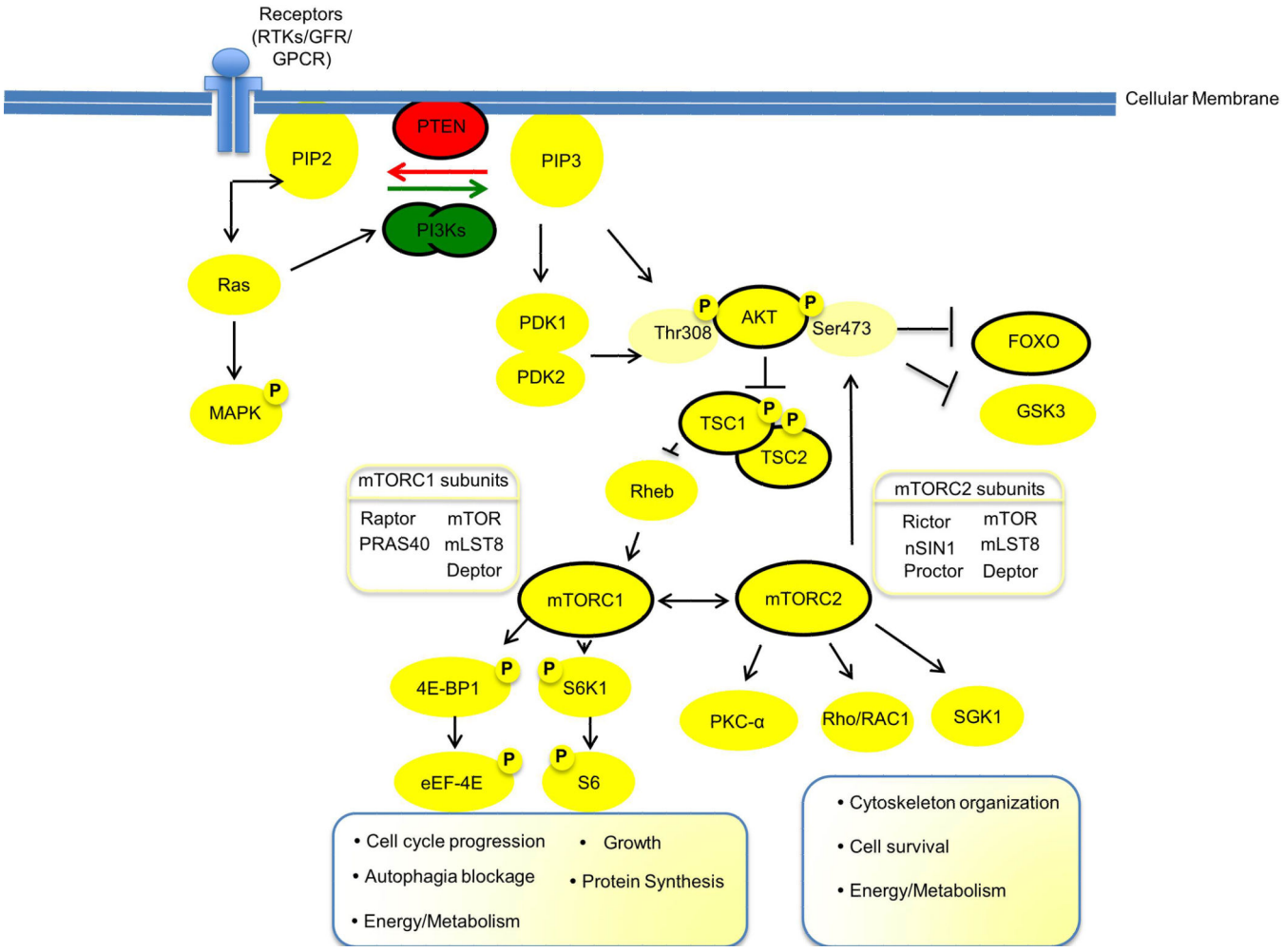

Figure 1.

The PI3K-PTEN-mTOR pathway. Activation of PI3K pathway via exogenous stimuli, endogenous signals and/or mutations promotes cancer formation, progression and resistance to therapy. The major signal modulators are PI3K in green and PTEN in red. The PI3Ks are shown as a simplified heterodimeric complex that combines the PI3K regulatory and catalytic subunits discussed in this review. As seen in the schematic representation, activated upstream cellular receptors (i.e. RTKs, GFRs, GPCRs) lead to activation of PI3Ks, which phosphorylate the membrane phospholipid substrate (i.e PIP2 into PIP3). PTEN is a phosphatase that opposes PI3K action; therefore, mutation or ablation of PTEN leads to PI3K/mTOR pathway over activation. Next, AKT is translocated to the cell membrane and is phosphorylated by PDK1 and PDK2. Once AKT is activated, it inhibits TSC1/TSC2 complex, which leads to RHEB inhibition. mTOR complexes (mTORC1 and mTORC2) collect the signals. mTORC1 activates downstream molecules. As a result, mTORC1 controls major processes, including cell cycle progression, growth, protein synthesis, lipid synthesis, and autophagy. mTORC2 functions are less understood, yet it is known to regulated, cell shape, cytoskeleton organization, cell survival, and cell metabolism. Therefore, PI3K/PTEN/mTOR pathway can control essential cancer cell processes, such as growth, invasion, metastases, and response to therapy. Black circles identify proteins that

were found mutated in HNSCC. RTK: receptors of tyrosine kinase; GFR: growth factors receptors; PI3K: phosphatidylinositol 3-kinase; PIP2: Phosphatidylinositol 4,5-bisphosphateor PtdIns(4,5)P2; PIP3: Phosphatidylinositol (3,4,5)-trisphosphate or PtdIns(3,4,5)P3; PTEN: Phosphatase and tensin homolog; MAPK: Mitogen-activated protein kinases; FOXO: forkhead box O; GSK3: glycogen-synthase-kinase-3; PKC: protein kinase C; Rac1: ras-related C3 botulinum toxin substrate 1; SGK1: serum/glucocorticoid regulated kinase 1; S6K: ribosomal protein S6 kinase; TSC1/2: tuberous sclerosis 1/2; 4E-BP1: eukaryotic initiation factor 4E-binding protein 1; eEF-4E: Eukaryotic translation initiation factor 4E; Rheb: Ras homolog enriched in brain; mTORC1/2: rapamycin-sensitive mTOR complex 1 and 2.

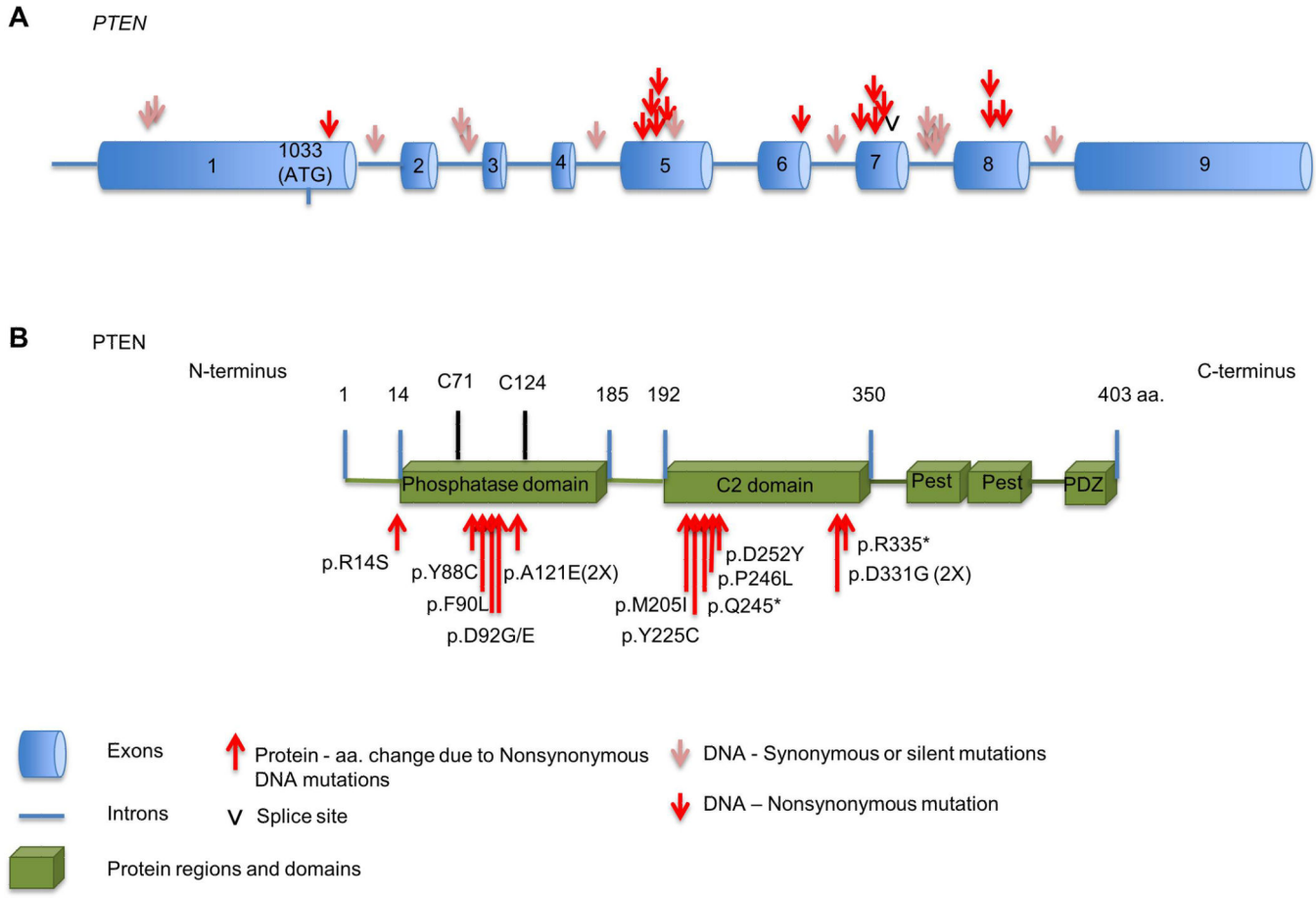

Figure 2.

PTEN mutations in HNSCC. **A**. Analyses of the mutations sites are shown in the exons and introns of the *PTEN* gene. **B**. Nonsynonymous mutations affecting the PTEN protein are clustered mostly within functional area of the phosphatase domain, between the active site cysteine residues 71 (C71) and 124 (C124). Additional mutations were in the C2-domain between the amino acids 192 and 350.

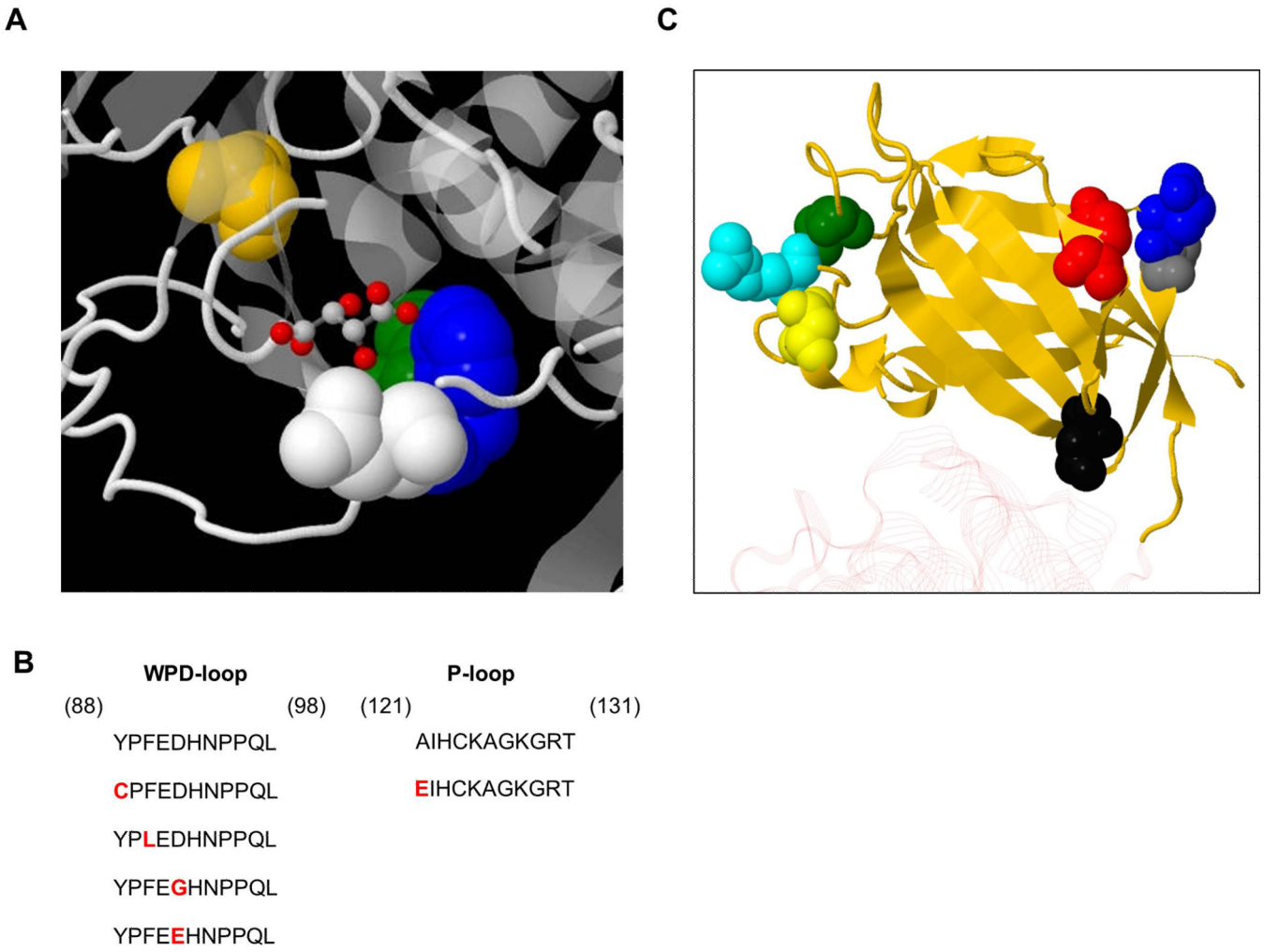

Figure 3.

Mutations of tumor suppressor PTEN in HNSCC. Crystal structure of PTEN protein is represented as ribbon diagrams. **A**. The functional active pocket of the phosphatase domain is shown. The mutated residues in the WPD-loop are highlighted in green (Y88), blue (F90), and white (D92). The mutated residue A121 is in yellow. **B**. Sequence of the WPD-loop and P-loop depicting the mutated residues within the walls of the active pocket present in the PTEN phosphatase domain. Mutated amino acid residues are in red. **C**. Mutated residues Q245 (in blue) and R335 (in cyan) in the C2 domain are nonsense mutations. Y225, P246, and D252 are shown in red, gray, and black respectively. M205 (in green) is in the Cβ 1/2 loop; and D331G (in yellow) is in the C2α loop.

Class of PI3Ks		Main lipid substrate	Catalytic Subunits		Regulatory subunit		Resulted lipid
			Protein	Gene	Protein	Gene	
I	IA	PIP2 or PI(4,5)P2	P110α	*PIK3CA*	p85α	*PIK3R1*	PIP3 or PI(3,4,5)P3
					P85β	*PIK3R2*	
			p110β	*PIK3CB*	p55α	*PIK3R1*	
			p100δ	*PIK3CD*	p50α	*PIK3R1*	
					p55γ	*PIK3R3*	
	IB		p100γ	*PIK3CG*	p101	*PIK3R5*	
					p87	*PIK3R6*	
II		PI	PI3K-C2α	*PIK3C2A*			PI(3,4)P2
		PI(4)P	PI3K-C2β	*PIK3C2B*			PI(3)P
			PI3K-C2γ	*PIK3C2G*			
III		PI	VPS34	*PIK3C3*	P150 (VPS15)	*PIK3R4*	PI(3)P

Figure 4.

Overview of the PI3K family members and their phospholipid substrates. PI3Ks constitute a large family of enzymes/oncogenes that is divided in the three classes as shown. Gene and protein names are paired by the catalytic and regulatory subunits that originate the PI3K heterodimers. Most common phospholipid substrate for each PI3K class is depicted.

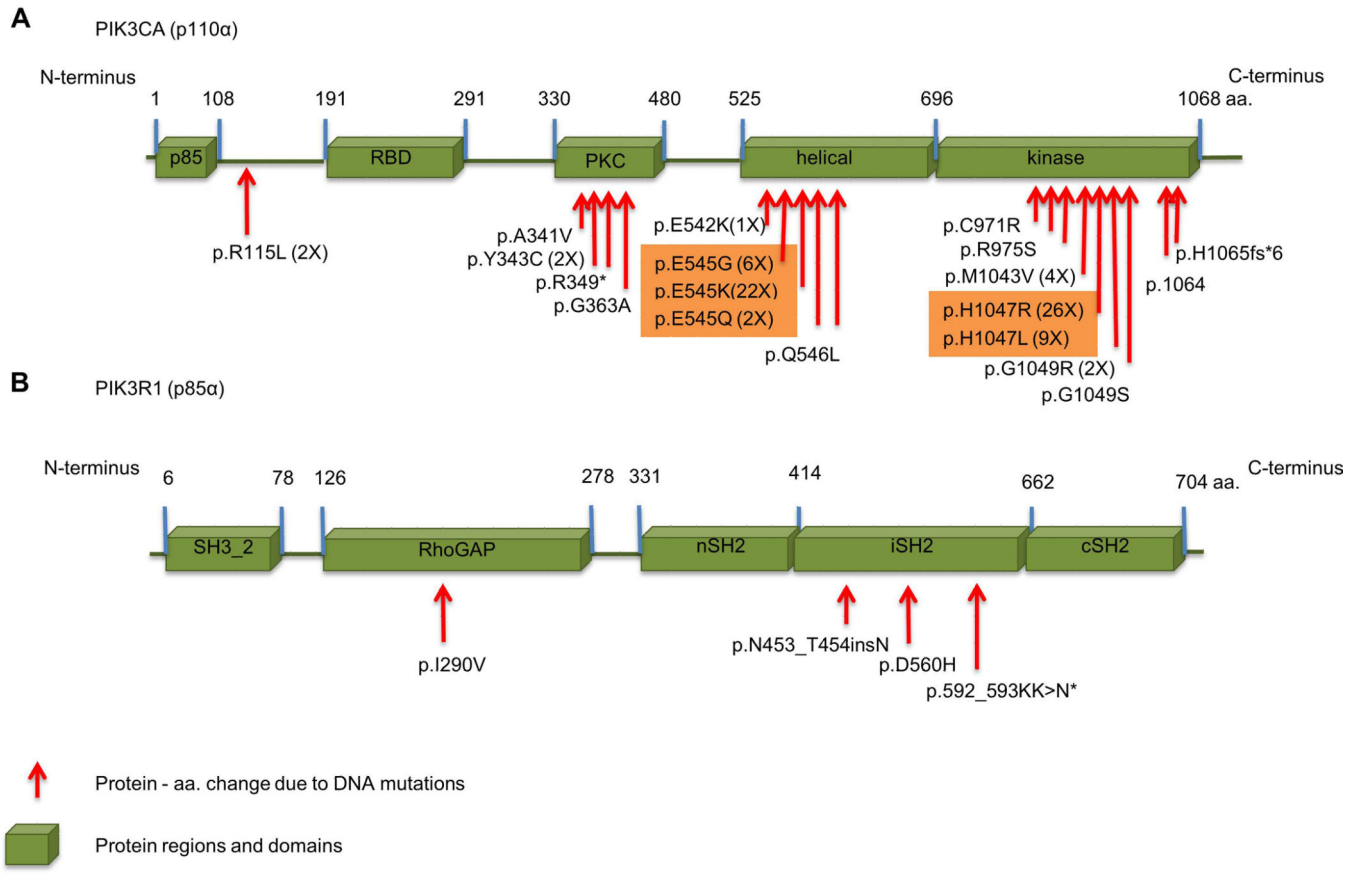

Figure 5.

Mutations observed in the PI3KCA and p85α. The amino acids residues show mutations in the indicated protein domains. **A**. Hot spot mutations of PI3KCA in HNSCC are E545K/G/K and p.H1047R/L as indicted in orange boxes. **B**. The site of mutations of regulatory subunit PIK3R1 (also known as p85α) are found within the RhoGAP-binding domain and the iSH2 domain.

AKT2 (putative phosphorylation site)

Species	Pospho-site	(-5) (5)
Homo sapiens (HNSCC mutation)	**Y351C**	GRLPF**C**NQDHE
Homo sapiens	Y351	GRLPFYNQDHE
Pan troglodytes	Y351	GRLPFYNQDHE
Macaca mulatta	Y351	GRLPFYNQDHE
Lupus familis	Y351	GRLPFYNQDHE
Mus musculus	Y351	GRLPFYNQDHE
Rattus norvegicus	Y351	GRLPFYNQDHE
Xenopus laevis	Y356	GRLPFYNQDHE
Brachydanio rerio	Y319	KPLQLYPN I SN
Dros. melanogaster	Y465	GRLPFYNRDHD
Caenorhab. elegans	Y392	GRLPFYSKDHN

Figure 6.

AKT2 mutation in HNSCC. Protein sequences of the putative phosphorylation site (Y) for AKT2 are conserved across species. The amino acid residue change found in the HNSCC (Y351C) is shown in red.

Table 1

Mutations of PI3K pathway components in HNSCC: PTEN, TSC, AKT, and mTOR complexes.

Gene Name	Gene alias	UniProt	Protein Name	Protein alias	Mutation type and UTR/Intron change	Amino Acid Change	Ref.
PTEN	*MMAC1, TEP1*	P60484	PTEN	MMAC1	Missense	p.A121E	23
					Missense	p.D252Y	2, 5
					Missense	p.D331G	23
					Missense	p.D92E	2; 5
					Missense	p.D92G	23
					Missense	p.F90L	23
					Missense	p.M205I	23
					Missense	p.P246L	2; 5
					Nonsense	p.R335*	2, 5
					Missense	p.Y225C	23
					Missense	p.Y88C	23
					Nonsense	p.Q245*	22
					Missense	p.R14S	5
					intron7(del. TT)		24
					c.e7_splice_site		2; 5
					intron 1		23
					intron 2		23
					intron 2		23
					intron 4		22
					intron 6		23
					intron 7		23
					intron 7 (39 base insertion)		22
					intron 7 (germiline)		24
					UTR		23
					UTR		23
TSC1	TSC, KIAA0243	Q92574	Hamartin	TSC1	Synonymous	p.P506P	2
					Nonsense	p.R245*	2; 5

Gene Name	Gene alias	UniProt	Protein Name	Protein alias	Mutation type and UTR/Intron change	Amino Acid Change	Ref.
TSC2	*TSC4*	P49815	Tuberin	TSC2	Nonsense	p.1415*	5
AKT2		P31751	AKT2	PKB-β, PKA-2, RAC-PK-β	Missense	p.Y351C	5
mTOR	*FRAP, FRAP1, FRAP2, RAFT1, RAPT1*	P42345	mTOR		Missense Missense	p.L2260H p.R1161Q	2; 5 2; 5
RICTOR	KIAA1999	Q6R327	RICTOR	hAVO3	Nonsense Missense	p.E1633* p.D175H	5 5
RPTOR	KIAA1303, RAPTOR	Q8N122	RAPTOR		Missense	p.P407L	5

Table 2

Mutations in the PI3K pathway components in HNSCC: PI3K family genes and proteins.

Gene Name	Gene alias	UniProt	Protein Name	Protein Alias	Mutation Type	Amino Acid Change	Ref.	Cell Line; Ref.
PIK3CA		P42336	PIK3CA	p100α, PI3Kα	Frameshift-Ins	p.1064insA	73	
					Missense	p.A341V	158	
					Missense	p.C971R	5	
					Missense	p.E542K	2, 5, 72, 158–160	SCC-6; 167–168
					Missense	p.E545G	2	HSC3; 73
					Missense	p.E545K	2, 5, 72,73, 159–165	HSC4; 72–73
					Missense	p.E545Q	82	SQ9G; 167
					Missense	p.G1049R	72, 163	
					Missense	p.G1049S	72	
					Missense	p.G363A	5	
					Missense	p.H1047L	2, 3	
					Missense	p.H1047R	2, 3, 5, 72, 160, 161,163	Detroit562; SUNE1; HONE1, CNE2; CAL-33; HSC2; HOC92; 71–73, 159, 166, 169–171.
					Frameshift	p.H1065fs*6	73	
					Missense	p.M1043V	72, 160	HOC719; 73
					Synonymous	p.P381P	159	
					Missense	p.Q546L	72	
					Missense	p.R115L	2	
					Nonsense	p.R349*	158	
					Missense	p.R975S	5	
					Synonymous	pT1025T	73	
					Missense	p.Y343C	159	
PIK3CB	*PIK3C1*	P42338	PIK3CB	p110β, PI3Kβ	Synonymous	p.P422P	2	
PIK3CD		O00329	PIK3CD	p110δ, PI3Kδ	Missense	p.T423S	5	
					Missense	p.T458S	2	
PIK3CG		P48736	PIK3CG	p110γ, PI3Kγ	Missense	p.A156V	2	

Gene Name	Gene alias	UniProt	Protein Name	Protein Alias	Mutation Type	Amino Acid Change	Ref.	Cell Line; Ref.
					Missense	p.G491E	2	
					Missense	p.L843H	2	
					Missense	p.P526S	2	
					Missense	p.R1021S	2	
					Missense	p.S446F	2	
					Missense	p.R178C	166	CAL-27; 166
PIK3R1	*GRB1*	P27986	PIK3R1	p85α/p55α/p50α, PI3Kα	In frame_Ins	p.453_454insN	2	
					Nonsense	p.592_593KK>N*	5	
					Missense	p.D560H	2	
					Missense	p.I290V	2	
PIK3R5		Q8WYR1	PIK3R5	***p101, FOAP-2, p101-PIK3***	Missense	p.A341V	5	
					Missense	p.E125G	5	
					Missense	p.E322K	5	
					Nonsense	p.E60*	5	
PIK3R6	*C17orf38*	Q5UE93	PIK3R6	p84, p87, p84 PIKAP, p87 PIKAP	Missense	p.R483H	2	
PIK3C2A		O00443	PIK3C2A	***PI3K-C2α***	Splice Site SNP		2	
					Synonymous	p.L226L	2	
PIK3C2B		O00750	PIK3C2B	PI3K-C2β, C2-PI3K	Missense	p.R564C	2	
PIK3C2G		O75747	PIK3C2G	PI3K-C2γ	Deletion	p.P129del	166	SCC-15; Detroit562; SCC-25; 166
					Missense	p.S1272L	5	
					Frameshift_Ins	p.T62fs	5	
					Missense	p.V656L	2	
PIKFYVE	PIP5K3, KIAA0981	Q9Y2I7	PIKfyve	PIP5K, PIP5K3, PIPkin-III	Missense Missense	p.H1851Q p.R1766P	2 2	