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## PIK3CA Mutations in Head and Neck Squamous Cell Carcinoma

Wanglong Qiu<sup>1</sup>, Frank Schönleben<sup>1</sup>, Xiaojun Li<sup>1</sup>, Daniel J. Ho<sup>1</sup>, Lanny G. Close<sup>1</sup>, Spiros Manolidis<sup>1</sup>, Boyce P. Bennett<sup>2</sup>, and Gloria H. Su<sup>1,2</sup>

<sup>1</sup> The Department of Otolaryngology/Head and Neck Surgery and the

<sup>2</sup> Department of Pathology, Columbia University College of Physicians and Surgeons, New York, NY 10032.

### Abstract

**Purpose**—Recent studies have reported high frequencies of somatic mutations in the *phosphoinositide-3-kinase, catalytic, alpha (PIK3CA)* gene in several human solid tumors. Although gene amplifications of *PIK3CA* have been reported in head and neck squamous cell carcinoma (HNSCC), small mutation of the gene has not been evaluated in HNSCC previously. In this study, we examined the mutation frequency of *PIK3CA* in HNSCC.

**Experimental Design**—More than 75% of the somatic mutations of *PIK3CA* are clustered in the helical (exon 9) and kinase domains (exon 20). To investigate the possible role of *PIK3CA* in HNSCC tumorigenesis, exons 1, 4, 5, 6, 7, 9, and 20 of the gene were analyzed by direct genomic DNA sequencing in 38 HNSCC specimens.

**Results**—We identified four missense mutations in the seven exons of *PIK3CA* from 38 HNSCC specimens (11%). Three of the four mutations, named H1047R, E542K and E545K respectively, have been previously reported as hot-spot mutations. The remaining novel mutation, Y343C, is identified at exon 4 nucleotide 1028 A → G. Three of the four mutations were shown to be somatic, while the fourth mutation (H1047R) was identified in a cell line. Interestingly, three of the four mutations identified were in pharyngeal cancer samples.

**Conclusions**—These data provide evidence that oncogenic properties of *PIK3CA* contributes to the carcinogenesis of human head and neck cancers, especially in pharyngeal cancer. A specific kinase inhibitor to *PIK3CA* may potentially be an effective therapeutic reagent against HNSCC or pharyngeal cancer in particular.

### The abbreviations used are

HNSCC, Head and neck squamous cell carcinoma; PCR, polymerase chain reaction; PI3K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol-3,4,5-triphosphate

## INTRODUCTION

The phosphatidylinositol 3-kinase (PI3K) signaling pathway regulates many normal cellular processes, such as cell proliferation, survival and apoptosis (1–3). Dysregulation or genetic aberration of components of this pathway, including *AKT*, *PTEN*, and *PIK3CA*, has been associated with cancer development (4–12).

To whom requests for reprints should be addressed: Dr. Su at the Department of Otolaryngology/Head and Neck Surgery, Columbia University College of Physicians and Surgeons, 1130 St. Nicholas Ave, ICRC 10-11, New York, NY 10032. Phone: 212-851-4624; E-mail: gs2157@columbia.edu..

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*PIK3CA* is located on chromosome 3q26.32 and encodes for the catalytic subunit p110 $\alpha$  of class IA PI3-kinase. It has been implicated to function as an oncogene in human cancer because of its elevated kinase activity and genomic amplification in tumor samples (7–12). Recently high frequencies of somatic mutations in the *PIK3CA* gene have been reported in several human cancer types, including colon, brain, stomach, breast, and ovary (13–18). More than 75% of these mutations are clustered in the helical (exon 9) and kinase domains (exon 20) of the gene (13). The three most frequently reported mutation hot spots in *PIK3CA*, named E542K, E545K and H1047R, have been shown to elevate its lipid kinase activity and lead to the activation of the downstream Akt signaling pathway (13,19). Interestingly, *PIK3CA* mutations and *PTEN* loss are nearly mutually exclusive, suggesting that the homeostasis of phosphatidylinositol-3,4,5-triphosphate regulated by both *PIK3CA* and *PTEN* is critical to carcinogenesis (20). This further evinced the importance of the PI3K pathway in the tumorigenesis of many cancer types.

Although the PIK3/AKT/PTEN pathway has been implicated in HNSCC (12,21–23), no genetic mutation of *PIK3CA* has been described to date. To investigate whether *PIK3CA* activating mutation is a common mechanism involved in the tumorigenesis of HNSCC, we analyzed for genetic alterations of the *PIK3CA* gene in 38 HNSCC specimens including eight cell lines by direct genomic DNA sequencing. Only exons 1, 4, 5, 6, 7, 9, and 20 of the gene were sequenced in these specimens because they covered the most common *PIK3CA* mutations previously observed in human cancer (13–17,24–26).

## MATERIAL AND METHODS

### Tissue samples and cell lines

Eight HNSCC cell lines, RPMI 2650, A-253, SW579, Detroit 562, FADU, CAL 27, SCC-15 and SCC-25, were purchased from American Type Culture Collection (Rockville, MD). The cell lines were maintained as recommended by ATCC.

Thirty frozen primary tumor samples and their corresponding match normal muscle specimens were obtained from the Tumor Bank facility of the Herbert Irving Comprehensive Cancer Center and Department of Otolaryngology/Head and Neck Surgery of the Columbia University Medical Center. Acquisition of the tissue specimens was approved by the Institutional Review Board of Columbia University Medical Center and performed in accordance with Health Insurance Portability and Accountability Act (HIPAA) regulations. Fresh-frozen tumor samples were dissected to ensure that the specimen contained at least 75% cancer cells. The cancer sites were nasal cavity (2), pharynx (6), larynx (10), oral cavity (8) and other sites (4). The patients' ages ranged from 40 to 85 years, average  $64.0 \pm 14.5$ . The grades of the tumors were moderately to poorly differentiated.

### PCR amplification and PCR product direct sequencing

Genomic DNAs were extracted from the cell lines and the frozen tissue samples using DNeasy tissue kit (Qiagen, CA). The procedures were performed according to the manufacturer's instructions.

Exons 1, 4, 5, 6, 7, 9, and 20 of *PIK3CA* gene were analyzed by PCR amplification of genomic DNA and PCR product direct sequencing. Genomic DNAs (40 ng per sample) were amplified with primers covering the entire coding region and the exon/intron boundaries of the desired exons (*PIK3CA*-E9F: 5'-ctgtgaatccagagggaaa-3'; *PIK3CA*-E9R: 5'-gcatttaatgtgccaactacca-3'; *PIK3CA*-E9FS: 5'-tccagaggggaaaaaatgaca-3', (13)). All gene sequencings were performed with ABI's 3100 capillary automated sequencers at the DNA facility of Columbia University Medical Center using previously published sequencing primers

(13). All samples found to have a genetic alteration in the target were subsequently sequenced in the reverse direction to confirm the mutation using the reverse PCR primers (13). The mutation was then further verified by sequencing of a second PCR product derived independently from the original template.

## RESULTS

### A novel sequence identified similar to *PIK3CA*

While sequencing for mutations using primers we had designed for exon 9, we were surprised to find an alteration at nucleotide 1634 A → C (E545A) in all the cases (Figures 1, 2). However, this nucleotide change of *PIK3CA* A1634C (E545A) always co-existed with another alteration of G → C at nucleotide 1658 and a base deletion at nucleotide 1659 (Figure 1). Subsequent sequencing analyses of the matching normal tissue specimens revealed that the same nucleotide changes occurred in both tumor and normal tissues (data not shown). This unusual result led us to blast search this PCR fragment (410-bp long) in the GenBank. We found two genomic DNA clones that contain fragments that are 97% (401/410) homologous to the exon 9 and its flanking intronic sequences. These two clones are located at chromosome 22q11.2 cat eye syndrome region (gi 5931525) and at chromosome 16 (gi 28913054) (Figure 2 and data not shown). Further comparisons of the sequences using the BLAST search revealed that both genomic clones on chromosome 22 and chromosome 16 contain sequences highly homologous to the exons 9, 11–13 and partial exon 10 of the *PIK3CA* gene (data not shown). An automatic computational analysis using the GNOMON gene prediction method predicted a protein that can be transcribed and translated from the chromosome 22 clone. The predicted protein (gi 51475436) is similar to the helical domain of the *PIK3CA* protein. However, this sequence homolog is likely to be a pseudogene since no RNA transcripts of the predicted protein can be detected by RT-PCR (data not shown).

This sequence homolog was probably not reported by previous publications because its detectability depends highly on primer designs. When we moved the PCR primer sites, used the primers published in the study by Samuels *et al.* (13), or increased the stringency of our PCR condition, all the nucleotide alterations including the so-called *PIK3CA* A1634C (E545A) “mutation” disappeared. We concluded that the A1634C (E545A) “mutation” observed in our hands was an artifact created by interferences from the sequence homolog.

### *PIK3CA* is activated by small mutation in HNSCC

Four missense mutations of the *PIK3CA* gene were identified in the 38 HNSCC specimens (Figure 3 and Table 1)-Two of the mutations were in the exon 9 (E545K, E542K), one was in the exon 20 (H1047Y) and one was in the exon 4 (Y343C). None of these mutations was detected in the corresponding normal tissues except for the H1047Y mutation, which was identified in HNSCC cell line Detroit 562. Three of the four *PIK3CA* missense mutations (E545K, E542K, and H1047R) are previously described hot-spot mutations (13). Functional studies showed that PI3-kinase carrying any one of the three hot-spot mutations is able to induce transformation in cultures of chicken embryo fibroblasts, and that the transforming activity of the mutant is correlated with increased lipid kinase activity and activation of the Akt signaling pathway (13,19). The mutation in the exon 4 nucleotide 1028 A → G, which leads to alteration at codon 343 TAC (Y) → TGC(C), has not been described before (Fig. 3).

Two other nucleotide alterations were also detected in the exonic regions of the *PIK3CA* gene (Table 1). One alteration, located at the exon 6 nucleotide 1173 A→G (codon 391 ATA (ILE) → ATG (Met)), was found in six HNSCC tumor specimens. This nucleotide alteration was also detected in the six matching normal tissues. A search of the SNP database revealed that A1173G is a known SNP (rs2230461) that has been validated by multiple PCR reactions and

genotype data. Thus, we conclude that this germline alteration represents a non-disease-causing polymorphism of *PIK3CA*. Another exonic alteration was also deemed a polymorphism because it occurred at exon 5 C1143G (P381P) without resulting in an amino acid change and was observed in both the tumor and normal samples of one tongue cancer patient. All of the polymorphisms observed in the intronic regions flanking the seven exons of *PIK3CA* examined in this study are listed in Table 2. These polymorphisms are unlikely to cause significant changes in the function of *PIK3CA*.

## DISCUSSION

The mutation frequency of *PIK3CA* has been reported at 32% in colon cancer, ~4–25% in gastric cancer, 8–40% in breast cancer, 5–27% in brain cancer, 4% in lung cancer, and 4–7% in ovarian cancer (13,16–18,25). In the present study we report 11% (4/38) of *PIK3CA* mutations in sporadic HNSCC. Interestingly, three out of the four cases with mutations are from the same organ site, pharynx (Table 1). Cancer of the pharynx is the 9<sup>th</sup> most common cancer worldwide (27). It is characterized as the following subsites: posterior pharynx, hypopharynx and lateral pharyngeal walls. A total of six pharyngeal squamous cell carcinoma cases were examined in this study, suggesting that as high as 50% (3/6) of pharyngeal tumor samples may harbor *PIK3CA* mutations. This data is supported by a previous report that showed chromosome 3q26 is amplified in 100% of nasopharyngeal carcinoma (22). However, from the present study we are unable to conclude the exact mutational frequency of *PIK3CA* in pharyngeal carcinomas and comment on which subtype of pharyngeal carcinomas (nasopharynx, oropharynx and hypopharynx) is targeted for *PIK3CA* mutation. Among our six pharyngeal samples, there was one oropharyngeal cancer sample, one hypopharyngeal cancer, and four that were not subtyped. More studies with larger sample sizes and various pharyngeal subtypes are necessary to further investigate these potentials.

Gene amplification is a more commonly observed mechanism of oncogene activation in HNSCC than small genetic mutation. *Cyclin D1* gene amplification has been observed in ~34–37% of HNSCC (28,29). *EGF* receptor gene amplification has been reported in 7–19% of HNSCC (30–32). In contrast, *RAS* mutation is relatively rare in HNSCC in comparison to other solid cancers- less than 6% in HNSCC vs. 99% in pancreatic cancer and 37–47% in colorectal cancer (33–39). Amplification of chromosome 3q26 is frequently observed in HNSCC and is linked to tumor progression and negatively correlated with clinical outcome (40–42). Gene amplification and overexpression of *PIK3CA* are observed in low to moderate dysplastic cases, but their increased frequencies are associated with transition to invasive cancer (21,23). Here we showed that gene amplification is not the only mechanism to activate *PIK3CA* in HNSCC. Small mutation and gene amplification both contribute to the activation of *PIK3CA* in HNSCC.

In summary, we report missense mutations of the *PIK3CA* gene in HNSCC (4/38, 11%). Among the four cases identified here, the Y343C mutation, which is located at *PIK3CA* exon 4 nucleotide 1028 A→G, is novel and has not been described in previous studies. Although the physiological significance of the novel mutation Y343C, which is located within the *PIK3CA* C2 domain, is not known, it has been shown that the C2 domain in the Class IB PI3K interacts primarily with the helical domain, and also interacts with the linker segment before the Ras-binding domain and with the C-terminal lobe of the catalytic domain (43). The C2 domain is often involved in Ca<sup>2+</sup>-dependent or Ca<sup>2+</sup>-independent phospholipids membrane binding. By analogy with enzymes like protein kinase C and cytosolic phospholipase A2, the C2 domain of ClassIB PI3K might participate in Ca<sup>2+</sup>-independent phospholipids membrane binding (43). Since mutations found in the C2 domain account for 7% of total *PIK3CA* mutations found in a study of 396 cancer samples (13), it will be worthwhile to determine the exact function of the C2 domain of Class 1A PI3K in future studies. The other three are hot-spot mutations (E545K, E542K, and H1047R) and all were found in pharyngeal cancer patients.

The smoking histories of the mutated patients are unknown. We did not find any significant correlation of the *PIK3CA* gene mutation to the gender or age of the patients.

Here we also report the discovery of a *PIK3CA* homolog. This homolog is almost identical to the exons 9, 11–13 and partial exon 10 of the *PIK3CA* gene and can be found on both chromosomes 16 and 22. However, we think that this sequence homolog is likely to be a pseudogene since no RNA transcripts of the predicted protein can be detected by RT-PCR. In our study, interferences from this sequence homolog had caused confusions by creating nucleotide alterations including the so-called *PIK3CA* A1634C (E545A) “mutation” (Figures 1, 2), which subsequently vanished with better primer designs and more stringent PCR conditions. Intriguingly, this A1634C (E545A) mutation has been previously reported in human cancers by two publications. One study described 11 cases with the A1634C (E545A) mutation out of 73 hepatocellular carcinomas (15). More recently this exact mutation was reported to contribute up to 88% (21/24) of the total *PIK3CA* mutations identified in ovarian cancer (24). This mutation was not described in other reports on *PIK3CA* mutation (13, 14, 16–18, 25, 26). In light of our discovery, it is important for future studies to be aware of the possible interference from the homologous sequences on chromosomes 22 and 16. Although we did not study exons 10–13 in our current study, potential artifacts there are also probable.

Our data confirm that *PIK3CA* is important to HNSCC tumorigenesis and provide evidence that small mutation can also contribute to oncogene activation of *PIK3CA* in HNSCC. Furthermore, our data suggest that *PIK3CA* gene mutations may be more involved in the carcinogenesis of a particular subset of human head and neck cancers (pharyngeal cancers) than others. The knowledge of the *PIK3CA*'s involvement in HNSCC is important because a specific kinase inhibitor could be considered as a future therapeutic option for HNSCC patients with *PIK3CA* mutations. Most HNSCC are diagnosed at advanced stage, and are usually unresectable despite significant surgical advances. Improvements in chemotherapy and radiotherapy in recent decades have not been translated into better prognosis of HNSCC patients (44). Recently kinase inhibitors such as Gleevec (Imatinib), Herceptin (Trastuzumab), and Iressa (Gefitinib) have been successfully developed for therapies in some cancer types (45). Since amplification and overexpression of the *PIK3CA* gene locus is an early oncogenic event of HNSCC tumorigenesis and is also correlated to invasion (21,23), abrogation of its oncogenic activities may conceivably slow or stop tumor progression. It is believed that such a selective small-molecule inhibitor against *PIK3CA* would have tremendous potential as a novel cancer chemotherapeutic for HNSCC (46). Our findings further supports *PIK3CA* as an important potential target in head and neck cancer for pathway-specific, kinase inhibitor based therapies.

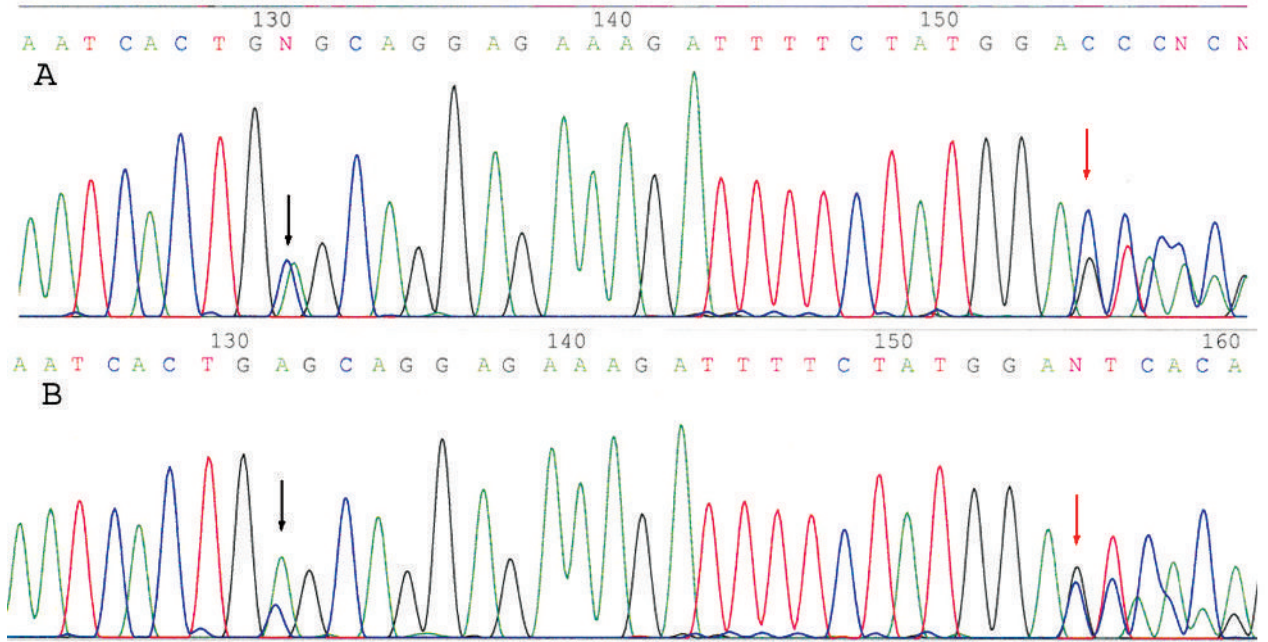
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**Figure 1. Identification of a sequence similar to *PIK3CA* on chromosomes 22 and 16**

Both A and B represent the so-called A1634C (E545A) “mutation” of the *PIK3CA* gene detected in all of our tumor samples. In our study, this “mutation” (black arrow) always co-existed with G1658C (red arrow) and a deletion of nucleotide 1659T. This sequencing profile was also detected in the matching normal specimens. We subsequently concluded that this abnormal profile is caused by the interference of a DNA sequence that is located at chromosome 22q11.2 cat eye syndrome region and chromosome 16 that are 97% homologous to the exon 9 of the *PIK3CA* gene.



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84126 acaacagttaattagcaatgtaaaagttttattgaaaatgtatttgctttttctgtaaatca
      |
      PIK3CA-E9F Primer
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      |
      PIK3CA exon 9
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      |
      ↓ nucleotide 1634
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      ↓ nucleotide 1658 & 1659
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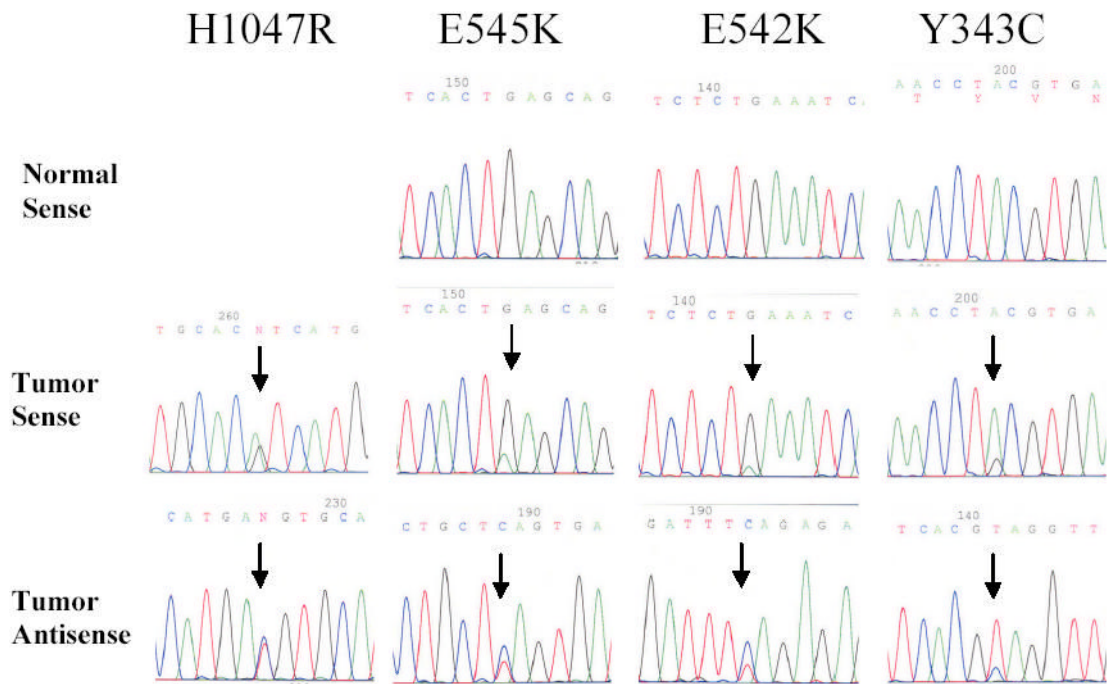
83161 aataactgaatttggctgatctcagcatgtttttaccatacctattggaataaataaagc
      |
84425 aataactgacttggctgatctcagcatgtttttaccatacctattagaataaatgaagc
      |
      hCT1640694-Ex9R Primer
83221 agaatttacatgatttttaactataaacattgcctttttaaaaacaatggttgtaaatt
      |
84485 agaatttacatgatttttaactataaacattgcctttttaaaaacaatggctgtaaatt
      |
      PIK3CA-E9R primer
83281 gatatttqtggaaaatcatactacattggtagttggcacattaatgctttttcttactc
      |
84545 gatatttgtagaaaatcatactacatttgtagttggcacattaatgcttttcttactc

83341 tgaattcctgatatgactttctttaggattgtttaaaatatt PIK3CA genomic DNA
      |
84605 tgaattcctgatatgactttctttaggattgtttaaaatatt Chr 22q11.2

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**Figure 2. The alignment of a PCR fragment of the *PIK3CA* gene containing exon 9 and its flanking intronic sequences with a human genomic DNA clone located at chromosome 22q11.2 Cat Eye Syndrome region (gi 5931525)**

The alignment shows that the homology between the two pieces of nucleotide sequences is 97% (401/410). Arrows mark the three nucleotide differences located inside the exon 9 coding region (in upper cases). The PCR primers designed by us (PIK3CA-E9F and PIK3CA-E9R) and Samuel *et al.* (hCT1640694-Ex9F and hCT1640694-Ex9R) are underlined.



**Figure 3. *PIK3CA* mutations found in HNSCC.** Three out of the four mutations (E545K, E542K, and Y343C) were confirmed to be somatic in sporadic HNSCC. The H1047R mutation was found in a HNSCC cell line.

**Table 1**  
Nucleotide alterations within the coding exons of *PIK3CA* identified in 38 HNSCC specimens

Cases	Exon	Nucleotide	Amino acid	Present in normal tissue	Tumor site (number)
Detroit 562	20	A3140G	H1047R	N/A	pharynx (1)
102T	9	G1624A	E542K	no	oropharynx (1)
109T	9	G1633A	E545K	no	hypopharynx (1)
182T	4	A1028G	Y343C	no	tongue (1)
80T	5	C1143G	P381P	yes	tongue (1)
6 cases	6	A1173G	I391M	yes	pharynx (2) oral (2) larynx(1) neck (1)

The nucleotide alterations are described according to the cDNA sequence with GenBank accession number NM\_006218.

**Table 2**  
Polymorphisms of *PIK3CA* found in 38 HNSCC specimens

Nucleotide position		Allele/allele frequency (number)		
IVS 1	+43 A>G	A/G (13)	A/A (14)	G/G (1)
	+130 insert TAT	heterozygosity (13)		homozygosity (1)
IVS 4	-69 G>T	G/T (15)	G/G (23)	T/T (0)
	-17 A>T	A/T (15)	A/A (16)	C/C (7)
IVS 5	+62 C>A	C/A (13)	C/C (13)	A/A (12)
	-38 T>C	T/C (4)	T/T (34)	C/C (0)
	+54 G>A	G/A (15)	G/G (14)	A/A (9)
	+307G>A	G/A (15)	G/G (14)	A/A (9)
IVS 7	+42 del TC	heterozygosity (1)		
IVS 9	+105 T>G	T/G (4)	T/T (34)	G/G (0)

The nucleotide alterations are described according to the genomic DNA sequences of *PIK3CA* (gi 8705172).