NIH Public Access
Author Manuscript
Int J Cancer. Author manuscript; available in PMC 2009 December 14.

Published in final edited form as:
Int J Cancer. 2008 March 1; 122(5): 1189–1194. doi:10.1002/ijc.23217.

Novel mutant-enriched Sequencing Identified High Frequency of *PIK3CA* Mutations in Pharyngeal Cancer

Wanglong Qiu[1], **Guo-Xia Tong**[2], **Spiros Manolidis**[1], **Lanny G. Close**[1], **Adel M. Assaad**[2], and **Gloria H. Su**[1,2]

[1] Departments of Otolaryngology and Head and Neck Surgery, Columbia University College of Physicians and Surgeons, New York, NY 10032

[2] Department of Pathology, Columbia University College of Physicians and Surgeons, New York, NY 10032

Abstract

We previously reported four *PIK3CA* mutations in 38 head and neck cancer samples; three of which were identified in six pharyngeal cancer samples. To determine the mutation frequency of *PIK3CA* in pharyngeal cancer, we studied 24 additional cases of pharyngeal squamous cell carcinoma in this study. Using both direct genomic DNA sequencing and novel mutant-enriched sequencing methods developed specifically for the three hot-spot mutations (H1047R, E545K and E452K) of *PIK3CA*, we detected five mutations of *PIK3CA* in the 24 pharyngeal cancers (20.8%). Three of the five mutations had been missed by the conventional sequencing method and were subsequently detected by novel mutant-enriched sequencing methods. We showed that the mutant-enriched sequencing method for the H1047R hot-spot mutation can identify the mutation in a mixed population of mutant and wild-type DNA sequences at 1:360 ratios. These novel mutant-enriched sequencing methods allow the detection of the *PIK3CA* hot-spot mutations in clinical specimens which often contain limited tumor tissues (i.e. biopsy specimens).

The data further supports that oncogenic *PIK3CA* may play a critical role in pharyngeal carcinogenesis, and the mutant-enriched sequencing methods for *PIK3CA* are sensitive and reliable ways to detect *PIK3CA* mutations in clinical samples. Because PIK3CA and its pathway are potential targets for chemotherapy and radiation therapy, and frequent somatic mutation of *PIK3CA* has been identified in many human cancer types (e.g. breast cancer, colorectal cancer), the abilities to detect *PIK3CA* mutations with enhanced sensitivities have great potential impacts on target therapies for many cancer types.

Keywords

mutant-enriched sequencing method; *PIK3CA* oncogene; hot-spot mutation; pharyngeal cancer; HNSCC

INTRODUCTION

Oral and pharyngeal cancer accounts for over half a million new cancer cases and 2,710,000 deaths worldwide per annum. It is the sixth most common malignant tumors in the world. In the United States alone, there were 30,990 estimated new cancer cases and 7,430 new deaths

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 10-04, New York, NY 10032. Phone: 212-851-4624; gs2157@columbia.edu.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

in the oral cavity and pharynx in 2006 [1]. Despite recent advances in surgical techniques and improvement in radiation therapy, the median survival of patients with head and neck cancer has changed little over the past few decades [2]. Thus, a better understanding of molecular and genetic feature of head and neck cancer would be critically helpful for the development of new methods for early diagnosis, monitoring, and targeting therapy, and the eventual improvement in the survival rate. Recently, significant progress has been achieved in the understanding of the molecular genetic events underlying the development of oral squamous cell carcinoma, which includes inactivation of multiple tumor suppressor genes (*p16, p53, p14,* and *FHIT*) and activation of oncogenes (*cyclin D1*and *EGFR*) [3, 4]. However, the molecular genetic profile of pharyngeal carcinogenesis is relatively less understood.

PIK3CA, which is a member of phosphatidylinositol 3-kinases (PI3Ks) family, has been demonstrated to function as an oncogene in some human cancer types *in vivo* and *in vitro* [5–8]. Recent studies have revealed a high frequency of somatic mutations at the *PIK3CA* locus in several human cancers [7, 9–12]. Approximately 80% of these somatic mutations clustered in the helical domain (exon 9) and kinase domain (exon 20) of *PIK3CA* 7. The three most frequent mutational spots of the *PIK3CA* gene, named H1047R, E545K, and E542K, are located at its exons 9 and 20. These mutations have been shown to elevate the *PIK3CA* oncogenic activities via the Akt signaling pathway [8, 13]. Increasing evidence has shown that *PIK3CA* plays an important oncogenic role in the tumorigenesis of many human cancer types, including human head and neck cancer [11].

The phosphatidylinositol 3′-kinase (PI3K) pathway is frequently activated in head and neck cancer through genomic amplification or activating mutation of the *PIK3CA* gene, or the loss of expression and/or function of the inhibitory Pten protein, that results in the activation of the Akt oncogenic signaling pathway [11, 14–17]. We have previously reported *PIK3CA* mutations in head and neck squamous cell carcinoma (4/38, 11%), and three of the reported *PIK3CA* mutations were identified in six pharyngeal squamous cell carcinoma samples in the study [11]. Due to the small sample size in that study, the true mutation frequency of *PIK3CA* in pharyngeal cancer could not be determined. To address that issue, in this present study, we aimed to investigate the mutation frequency of the *PIK3CA* gene in additional 24 pharyngeal cancer specimens.

MATERIAL AND METHODS

Patients and tissue samples

Twenty-four cases of paraffin-embedded pharyngeal cancer blocks were obtained from the Department of Pathology of the Columbia University Medical Center (CUMC). The acquisition of the tissue specimens was approved by the Institutional Review Board and performed in accordance with Health Insurance Portability and Accountability Act (HIPAA) regulations. The 24 cases studied included five surgical resection specimens and nineteen cases of small biopsy specimens. They came from five female and nineteen male patients, with ages ranging from 38 to 78 years-old (average 57.9 ± 12.2 years-old). Eleven were heavy smokers (more than 40 packs per year), two were moderate smokers, two had no smoking and had only occasional alcohol use, and the remaining nine patients' history was not available. Two of the 11 heavy smokers also had heavy alcohol consumption and one abused cocaine. One patient was a HIV carrier. All patients were diagnosed as squamous cell carcinomas of the pharynx. The grade of cancer in these patients was four well-, 18 moderately-, and two poorly-differentiated. The cases were reviewed by two pathologists and the diagnosis confirmed.

Five 10μm thickness sections were cut for each case and genomic DNA was extracted from the tumor tissues using QIAmp DNA Kit (QIAGEN Inc., Valencia, CA). The procedures were

NIH-PA Author Manuscript

performed according to the manufacturer's instructions for purification of genomic DNA from paraffin-embedded tissue.

Conventional genomic sequencing

Exons 9 and 20 of *PIK3CA* gene were analyzed by PCR amplification of genomic DNA (40ng each) and direct sequencing of the PCR products. New PCR primers were designed for this study to allow more efficient amplifications of genomic DNA from paraffin-embedded tissues. Primers for exon 9 were also designed to avoid interference from a homologous pseudogene located on chromosome 22q11.2 cat eye syndrome region [11]. The primers for the *PIK3CA* exons 9 and 20 are PIK-E9F: CCAGAGGGGAAAAATATGACA; PIK-E9R: CATTTTAGCACTTACCTGTGAC; PIK-E20F: CATTTGCTCCAAACTGACCA; PIK-E20R: TGAGCTTTCATTTTCTCAGTTATCTTTTC. Before sequencing, PCR products were purified using the Geneclean Turbo Nucleic Acid purification Kit (Qbiogene, Irvine, CA). Finally, purified DNA fragments were sequenced using the corresponding forward PCR primers. Samples found to have a genetic alteration in the target gene were subsequently sequenced in the reverse direction to confirm the mutation using the reverse PCR primers. The mutation was then further verified by sequencing of a second PCR product derived independently from the original template. All sequencings were performed with ABI's 3100 capillary automated sequencers at the DNA facility of the CUMC [11].

Mutant-enriched sequencing for detecting *PIK3CA* mutations, H1047R, E545K, and E542K

To detect the *PIK3CA* hot-spot mutation A3140G (H1047R), each sample (40ng of genomic DNA) was first amplified using outer primers PIK-E20OF (GACATTTGAGCAAAGACCTGAA) and PIK-E20OR (ATCAAACCCTGTTTGCGTTT) for 30 cycles. After this first round of PCR, 2μl from each PCR product were digested with 2μl of restriction enzyme BsaBI (10U/ul, New England BioLabs, Ipswich, MA) in a total of 50 μl volume at 60°C overnight. Then 2μl of the digest were used for the second round of PCR for 40 cycles. The primers for the second PCR are PIK-E20IF (CATTTGCTCCAAACTGACCA) and PIK-E20IR (TGAGCTTTCATTTTCTCAGTTATCTTTTC). Each PCR product with the correct size was purified for DNA sequencing using the same primers as for the second PCR (PIK-E20IF or PIK-E20IR) (Fig. 1A).

For mutant-enriched sequencing of *PIK3CA* exon 9 hot-spot mutation, G1633A (E545K), the procedure is similar to the one described above for the hot-spot mutation A3140G, except for the enzyme and primers used. A mismatch primer PIK-E9MF (TCTACACGAGATCCTCTCTCTGTAATCTC) was used as the forward primer for both rounds of PCR. The reverse primers for the first and second PCR were respectively PIK-E9OR (GCATTTAATGTGCCAACTACCA) and PIK-E9IR (CTGAGATCAGCCAAATTCAGTTATTTTTTC). The restriction enzyme digestion was performed with Hpy188I at 37°C overnight. The reverse PCR primer PIK-E9R was also used as the DNA sequencing primer (Fig. 1B).

For the hot-spot mutation at exon 9, G1624A (E542K), the PCR strategy of mutant-enriched sequencing is the same as the one described above for the hot-spot mutation G1633A (E545K), in which a mismatch primer is designed to create a unique restriction enzyme site EcoRI in the *PIK3CA* exon 9 region. The mismatch primer PIK-2E9MR (CATAGAAAATCTTTCTCCTGCTCAGTGAAT) was used as the reverse primer for both rounds of PCR. The forward primers for the first and second PCR were respectively PIK-2E9OF (GATTGGTTCTTTCCTGTCTCTG) and PIK-2E9IF (TTGCTTTTTCTGTAAATCATCTGTG). The restriction enzyme EcoRI digestion was

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

performed at 37°C overnight. The forward PCR primer PIK-2E9IF was also used as the DNA sequencing primer (Fig. 1C).

The PCR condition for all the PCR reactions is 94°C, 2 minutes; (94°C, 30 seconds; 60°C, 30 seconds; 72°C, 30 seconds) × 40 cycles; 72°C, 5 minutes.

RESULTS

Conventional DNA sequencing detected *PIK3CA* mutations only in surgically resected but not in biopsy specimens of pharyngeal cancer

We initially screened for *PIK3CA* mutation in 24 cases of pharyngeal squamous cell carcinoma samples using the direct genomic sequencing method that we had applied to our previous study on HNSCC [11]. Because most pharyngeal cancers are treated non-surgically (i.e. concurrent chemoradiation), 19 of our specimens were biopsy tissues. Two mutations of *PIK3CA* were identified in the five resection samples and none in the biopsy specimens. One was *PIK3CA* hot-spot mutation G1633A (E545K) (Fig. 2A). The other mutation, was a missense mutation in exon 20 nucleotide 3127 A→ G, led to a codon 1043 ATG (Met)→GTG (Val) substitution (Fig. 2B). This missense mutation of *PIK3CA* has been reported previously [18]. Both mutations were not detected in the surrounding normal tissues, thus both were somatic mutations.

Mutant-enriched sequencing identified *PIK3CA* hotspot mutation H1047R in samples screened negative by conventional DNA sequencing approach

Conventional DNA sequencing method only recognizes the mutant DNA if it is present in more than 10 percent of a mutant/wild-type mixed population in primary tumor tissue [19, 20]. Therefore we hypothesized that the low tumor to normal cell ratio in our biopsy specimens may have generated some false negative results and contributed to the lower frequency of *PIK3CA* mutation observed in the current study (2/24, 8.3%) than our previous report (3/6, 50%) [11]. The unexpectedly low mutation frequency of *PIK3CA* in the biopsy samples might also have been caused by the overall low or poor DNA contents available in these tissues. We set out to develop a mutant-enriched sequencing method to detect hot-spot mutations of *PIK3CA* in specimens with low tumor DNA contribution, such as biopsy samples.

Mutant-enriched sequencing methods generally involve a first-round PCR, restriction enzyme digest, and a second-round PCR. They selectively amplify the mutant copy of a target gene in the second round of PCR by reducing the wild-type copy number via a restriction enzyme digestion that is specific for the wild-type sequence after the first round PCR. Thus, mutant-enriched DNA sequencing is particularly valuable when the ratio of mutant DNA is expected to be low. In our method of mutant-enriched sequencing for the detection of *PIK3CA* exon 20 hotspot mutation A3140G (H1047R), we took advantage of a natural restriction enzyme site on the wild-type sequence that can be recognized and digested by enzyme BsaBI (cuts GATNNNNATC). This enzyme site is destroyed when the DNA is mutated, thus enzyme BsaBI exclusively digests the wild-type *PIK3CA* exon 20 DNA, but not the mutant A3140G (H1047R) sequence (Fig. 1A).

The feasibility of our method was first investigated in a head and neck cancer cell line, Detroit 562, which harbors the H1047R mutation [11]. As shown in Figure 3A1–2, both the mutant and wild-type peaks were observed at 1:1 ratio as expected using conventional genomic sequencing (Fig. 3A1), and the wild-type peak was entirely eliminated when our mutant-enriched sequencing method was applied (Fig. 3A2). To determine the sensitivity of our assay, we made a series of dilutions of the genomic DNA from mutant cell line Detroit 562 with DNA from another cell line that has two wild-type *PIK3CA* alleles. When the ratio of mutant and wild-type DNA copies reached beyond 1: 360, the mutant peak could still be recognized by mutant-

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

enriched sequencing (Fig. 3A2). In contrast, with conventional PCR-sequencing, the mutant peak disappeared when the ratio of mutant and wild-type DNA reached 1:18. This indicated that mutant-enriched sequencing was at least twenty fold more sensitive than direct genomic sequencing.

Using this mutant-enriched sequencing method, two additional mutations of the *PIK3CA* gene were identified in these 24 pharyngeal cancer samples (data not shown and Table 1). This result supported our hypothesis that the low frequency of *PIK3CA* mutation detected in clinical pharyngeal cancer biopsy samples by the conventional DNA sequencing method was partially caused by the contamination of normal cells.

Mutant-enrich sequencing for *PIK3CA* exon 9 hotspot mutation E545K

For mutant-enriched sequencing of *PIK3CA* exon 9 hotspot mutation, G1633A (E545K), mismatch primer (PIK-E9MF) was designed to introduce two A→ T nucleotide mismatches in the forward primer to create a unique restriction enzyme site Hpy188I (TCNGA) (Fig. 2B), because there is no natural unique restriction enzyme site specific for the wild-type but not the mutant DNA sequences at this hot-spot. Using a patient's tumor DNA with known *PIK3CA* E545K mutation (patient No. 4, Table 1), we showed that using our mutant-enriched sequencing method, only the mutant peak remained while the more prominent wild-type peak observed in the conventional sequencing assay disappeared (Fig. 3B1–2). Applying this powerful method, we screened the 24 pharyngeal cancer samples. We did not uncover any additional cases at this hot-spot mutation.

Mutant-enrich sequencing for *PIK3CA* exon 9 hotspot mutation E542K (G1624A) and a non-hot-spot mutation E542G (A1625G)

We applied the same mismatch PCR strategy to enrich the mutant allele of *PIK3CA* hotspot mutation E542K. A restriction enzyme EcoRI site was introduced by the mismatch primer PIK-2E9R. EcoRI digestion disrupted the wild-type *PIK3CA* DNA, but not the mutant of *PIK3CA* E542K sequences (Fig. 1C).

This mutant-enriched sequencing method was tested in a previously reported patient sample with a known *PIK3CA* E542K mutation [11]. We showed that only the mutant peak remained while the corresponding wild-type peak completely vanished when the mutant-enriched sequencing was applied (Fig. 3C1–2). Interestingly, this method can detect not only the E542K (G1624A) mutation, but also a previously described non-hot-spot E542G (A1625G) mutation [13] (data not shown). We screened the 24 specimens of pharyngeal cancer and an additional case of *PIK3CA* E542K (G1624A) mutation was identified (Fig. 3C3–4).

Five mutations of the *PIK3CA* gene were identified in the 24 pharyngeal cancer samples

Five mutations of *PIK3CA* were identified in the 24 cases of pharyngeal cancer by the combination of conventional sequencing and mutant-enriched sequencing methods (Table 1). Four of the five mutations could have been identified by the mutant-enriched sequencing methods alone because they were hot-spot mutations (4/5, 80%), but only two were detectable by the conventional sequencing method (2/5, 40%). Two patients with *PIK3CA* mutations were not smokers and only drank alcohol occasionally, while one was a heavy smoker and drank alcohol occasionally (Table 1). There is no apparent association between *PIK3CA* mutation and smoking or alcohol consumption. There is also no apparent association between *PIK3CA* mutation and the degree of tumor cell differentiation (Table 1).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

DISCUSSION

Previously we reported *PIK3CA* mutations in head and neck cancers (4/38, 10%), three of the four were identified in pharyngeal cancer samples (3/6, 50%) [11]. However, whether *PIK3CA* can be used as a potential biomarker for diagnosis and molecular target therapy in pharyngeal cancer was unclear, due to the small sample number available at the time. Therefore, we decided to investigate the frequency of *PIK3CA* mutation in pharyngeal cancer using 24 additional samples.

Initially, only two mutations of *PIK3CA,* including a hotspot mutation E545K and a missense mutation in exon 20 nucleotide 3127 A→ G, were found in five surgically resection specimens and none in 19 biopsy specimens by the conventional genomic sequencing method. We attributed this low mutation frequency to the quality of clinical biopsy samples. Clinical biopsy samples often contain small numbers of tumor cells mixed with a large population of normal cells. The mutant DNA is often missed by the conventional PCR- sequencing method. To increase the sensitivity of detecting *PIK3CA* gene mutation, we developed novel mutant-enriched DNA sequencing methods for its three hot-spot mutations, H1047R, E545K and E542K. The three hot-spot mutations account for 78.6% of all *PIK3CA* mutations reported [7, 21]. We were able to show that mutant-enriched sequencing can identify the H1047R mutant DNA in a mixed population with wild-type DNA at sensitivity of 0.0028 (1 mutant: 360 wild-type DNA copies). Using this mutant-enriched sequencing method for H1047, we found two additional mutations in these pharyngeal cancer samples. An additional mutation was identified by the mutant-enriched sequencing protocol for hotspot mutation H542K (G1624A). Thus, a total of five *PIK3CA* mutations were identified in 24 cases of pharyngeal cancer in combination of regular DNA sequencing and mutant-enriched sequencing. It is important to note that four of the five mutations were hot-spot mutations and could have been identified by the mutant-enriched sequencing methods alone (4/5, 80%), but only two were detectable by the conventional sequencing method (2/5, 40%). This means that the ability to detect *PIK3CA* mutations increased by 200% when the mutant-enriched sequencing methods are utilized. The 80% detection rate is within the expectation, because the three hot-spot mutations account for ~80% of total *PIK3CA* mutations. Two patients with *PIK3CA* mutations did not have a history of smoking or alcohol-abuse. This suggests that *PIK3CA* mutation might be a critical cause for those pharyngeal cancer patients without history of smoking and alcohol consumption. *PIK3CA* mutation was not found associated with the degree of differentiation in the current study.

The primers for the mutant-enriched sequencing analyses of the *PIK3CA* exon 9 hotspot mutations were designed to avoid interference from a homologous pseudogene located on chromosome 22q11.2 cat eye syndrome region [11]. At least one of the two primers for each PCR amplification contains mismatched base pairs to the pseudogene. Those mismatched nucleotides in either the forward and/or reverse primers seemed sufficient to prevent PCR amplification of the pseudogene in our mutant-enriched sequencing analyses. This was supported by the fact that we never observed the A1634C change in our samples. The A1634C change was mistaken as a mutation in previous literatures, when in fact it is only one of the dissimilarities between the pseudogene and *PIK3CA* at Exon 9, nucleotide 1634 (it’s A for *PIK3CA* and C for the pseudogene) [11]. We also did not observe the frameshift change that had also been associated with the amplification of the pseudogene [11].

Comparing to mutant-selective PCR and restriction fragment length polymorphism (RFLP) analysis [19, 20], we believe that our mutant-enriched sequencing method is more sensitive and specific because: 1) there is the possibility of the non-specific digestion by the restriction enzyme when confirming the mutation by PCR-RFLP; 2) small amount of mutant DNA after digestion may not be enough to be visualized on an agarose gel in the PCR-RFLP analysis.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

The mutant-enriched sequencing method directly displays the exact nucleotide sequence; 3) the mutant-enrich sequencing method is not limited by available restriction enzyme sites. A unique enzyme site could be introduced by mismatch PCR, as we have done for the detection of *PIK3CA* exon 9 hot-spot mutations E545K and E542K. Thus, the mutant-enriched sequencing method is more superior to both the PCR-RFLP analysis and the conventional genomic sequencing assay for detecting hot-spot mutations. This method is particularly valuable in clinical applications where tumor samples are often mixed with a large population of normal cells.

Our current study concluded that *PIK3CA* mutation frequency in pharyngeal cancer is ~21% (5/24). A recent study of *PIK3CA* mutation in nasopharyngeal carcinoma reported two mutations in six cell lines and but none was identified in 40 clinical samples (4.3% or 2/46) [15]. It is possible that the low tumor to normal DNA ratio in their clinical samples might have contributed to the negative finding in that study. Thus, a more sensitive assay could have improved the detection of *PIK3CA* mutations in such clinical samples where limited amounts of tumor DNA were available.

In conclusion, sensitive mutant-enriched sequencing methods were developed to detect the three hotspot mutations of the *PIK3CA* gene in clinical tumor samples. This novel detection method can detect approximately 80% of all *PIK3CA* mutations [21] and is valuable in clinical applications particularly in samples with little tumor cell contribution (i.e. biopsy samples) or with tumor subclones that usually go undetected by the conventional sequencing method because of their minor cellular populations. Several studies have shown that PIK3CA and its pathway are potential targets for chemotherapy or radiation therapy, including target therapies for EGFR, Her-2, mTOR, and Akt [22, 23]. Therefore, the clinical applications of the mutant-enriched sequencing methods would have great potential impacts on early detection and target therapy for many cancer types harboring frequent *PIK3CA* mutations (*e.g.* breast cancer, colorectal cancer).

Acknowledgments

This work was supported by the NCI Temin Award CA095434 and the NCI R01 CA109525.

The abbreviations used are

HNSCC	Head and neck squamous cell carcinoma
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase

References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ. Cancer statistics. CA Cancer J Clin 2006;56:106–30. [PubMed: 16514137]
2. Carvalho AL, Nishimoto IN, Califano JA, Kowalski LP. Trends in incidence and prognosis for head and neck cancer in the United States: a site-specific analysis of the SEER database. Int J Cancer 2005;114:806–16. [PubMed: 15609302]
3. Forastiere A, Koch W, Trotti A, Sidransky D. Head and neck cancer. N Engl J Med 2001;345:1890–900. [PubMed: 11756581]
4. Williams HK. Molecular pathogenesis of oral squamous carcinoma. Mol Pathol 2000;53:165–72. [PubMed: 11040937]
5. Ma YY, Wei SJ, Lin YC, Lung JC, Chang TC, Whang-Peng J, Liu JM, Yang DM, Yang WK, Shen CY. PIK3CA as an oncogene in cervical cancer. Oncogene 2000;19:2739–44. [PubMed: 10851074]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

6. Shayesteh L, Lu Y, Kuo WL, Baldocchi R, Godfrey T, Collins C, Pinkel D, Powell B, Mills GB, Gray JW. PIK3CA is implicated as an oncogene in ovarian cancer. Nat Genet 1999;21:99–102. [PubMed: 9916799]
7. Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, Yan H, Gazdar A, Powell SM, Riggins GJ, Willson JK, Markowitz S, et al. High frequency of mutations of the PIK3CA gene in human cancers. Science 2004;304:554. [PubMed: 15016963]
8. Kang S, Bader AG, Vogt PK. Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic. Proc Natl Acad Sci U S A 2005;102:802–7. [PubMed: 15647370]
9. Lee JW, Soung YH, Kim SY, Lee HW, Park WS, Nam SW, Kim SH, Lee JY, Yoo NJ, Lee SH. PIK3CA gene is frequently mutated in breast carcinomas and hepatocellular carcinomas. Oncogene 2005;24:1477–80. [PubMed: 15608678]
10. Campbell IG, Russell SE, Choong DY, Montgomery KG, Ciavarella ML, Hooi CS, Cristiano BE, Pearson RB, Phillips WA. Mutation of the PIK3CA gene in ovarian and breast cancer. Cancer Res 2004;64:7678–81. [PubMed: 15520168]
11. Qiu W, Schonleben F, Li X, Ho DJ, Close LG, Manolidis S, Bennett BP, Su GH. PIK3CA mutations in head and neck squamous cell carcinoma. Clin Cancer Res 2006;12:1441–6. [PubMed: 16533766]
12. Schonleben F, Qiu W, Ciau NT, Ho DJ, Li X, Allendorf JD, Remotti HE, Su GH. PIK3CA mutations in intraductal papillary mucinous neoplasm/carcinoma of the pancreas. Clin Cancer Res 2006;12:3851–5. [PubMed: 16778113]
13. Samuels Y, Velculescu VE. Oncogenic mutations of PIK3CA in human cancers. Cell Cycle 2004;3:1221–4. [PubMed: 15467468]
14. Garcia-Rostan G, Costa AM, Pereira-Castro I, Salvatore G, Hernandez R, Hermsem MJ, Herrero A, Fusco A, Cameselle-Teijeiro J, Santoro M. Mutation of the PIK3CA gene in anaplastic thyroid cancer. Cancer Res 2005;65:10199–207. [PubMed: 16288007]
15. Or YY, Hui AB, To KF, Lam CN, Lo KW. PIK3CA mutations in nasopharyngeal carcinoma. Int J Cancer 2006;118:1065–7. [PubMed: 16114017]
16. Pedrero JM, Carracedo DG, Pinto CM, Zapatero AH, Rodrigo JP, Nieto CS, Gonzalez MV. Frequent genetic and biochemical alterations of the PI 3-K/AKT/PTEN pathway in head and neck squamous cell carcinoma. Int J Cancer 2005;114:242–8. [PubMed: 15543611]
17. Woenckhaus J, Steger K, Werner E, Fenic I, Gamerdinger U, Dreyer T, Stahl U. Genomic gain of PIK3CA and increased expression of p110alpha are associated with progression of dysplasia into invasive squamous cell carcinoma. J Pathol 2002;198:335–42. [PubMed: 12375266]
18. Kozaki K, Imoto I, Pimkhaokham A, Hasegawa S, Tsuda H, Omura K, Inazawa J. PIK3CA mutation is an oncogenic aberration at advanced stages of oral squamous cell carcinoma. Cancer Sci 2006;97:1351–8. [PubMed: 17052259]
19. Levi S, Urbano-Ispizua A, Gill R, Thomas DM, Gilbertson J, Foster C, Marshall CJ. Multiple K-ras codon 12 mutations in cholangiocarcinomas demonstrated with a sensitive polymerase chain reaction technique. Cancer Res 1991;51:3497–502. [PubMed: 1675933]
20. Nakahori S, Yokosuka O, Ehata T, Chuang WL, Imazeki F, Ito Y, Ohto M. Detection of hepatitis B virus precore stop codon mutants by selective amplification method: frequent detection of precore mutants in hepatitis B e antigen positive healthy carriers. J Gastroenterol Hepatol 1995;10:419–25. [PubMed: 8527708]
21. Bader AG, Kang S, Zhao L, Vogt PK. Oncogenic PI3K deregulates transcription and translation. Nat Rev Cancer 2005;5:921–9. [PubMed: 16341083]
22. Rogers SJ, Box C, Harrington KJ, Nutting C, Rhys-Evans P, Eccles SA. The phosphoinositide 3-kinase signalling pathway as a therapeutic target in squamous cell carcinoma of the head and neck. Expert Opin Ther Targets 2005;9:769–90. [PubMed: 16083342]
23. Kim DW, Huamani J, Fu A, Hallahan DE. Molecular strategies targeting the host component of cancer to enhance tumor response to radiation therapy. Int J Radiat Oncol Biol Phys 2006;64:38–46. [PubMed: 16377414]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Fig. 1. The schematic of the mutant-enriched sequencing methods for detecting *PIK3CA* mutations, H1047R, E545K and E542K

A. The protocol for detecting*PIK3CA* mutation H1047R. Enzyme BsaBI specifically cuts the wild-type sequences of the exon 20, but not the mutant copies with A3140G nucleotide alteration. After digesting the first PCR product with the enzyme BsaBI, the second PCR selectively amplifies the mutant copies. *B.* A unique restriction enzyme site Hpy188I was introduced by mismatch PCR for detecting *PIK3CA* mutation E545K. The mismatch primer (PIK-E9MF) has two A→ T nucleotide substitutions in the forward primer to create a unique enzyme site Hpy188I in the wild-type sequences of the *PIK3CA* exon 9, but not the mutant sequences. *C.* The mismatch primer PIK-2E9MR was designed to create a unique restriction

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

enzyme site EcoRI for enriching *PIK3CA* mutation E542K (G1624A) and E542G (A1625G) with the similar strategy for the hot-spot mutation E545K.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

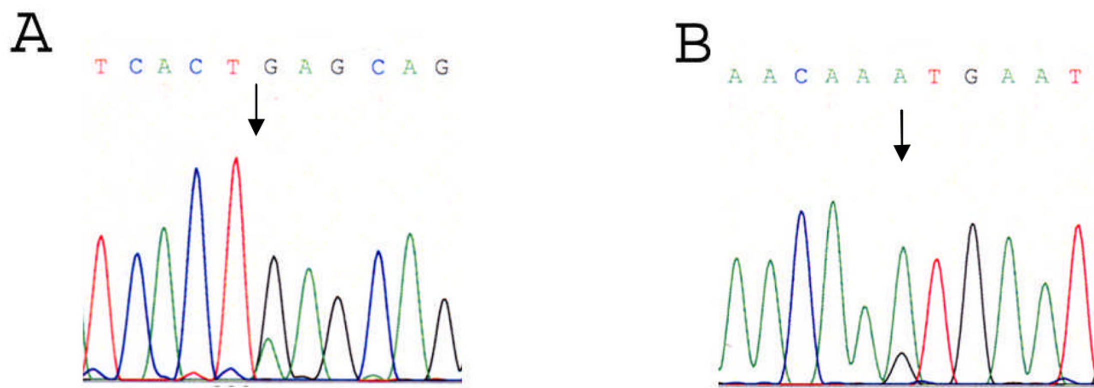

Fig. 2. Two mutations of *PIK3CA* were identified in 24 pharyngeal cancer samples using conventional DNA sequencing

A. Hot-spot mutation G1633A (E545K) of *PIK3CA* gene causes a change in amino acid codon from 545 GAG (glutamic acid) to AAG (lysine). *B*. A point mutation at *PIK3CA* exon 20 nt 3127A→G leads to codon substitution from 1043 ATG (Met) to GTG (Val).

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

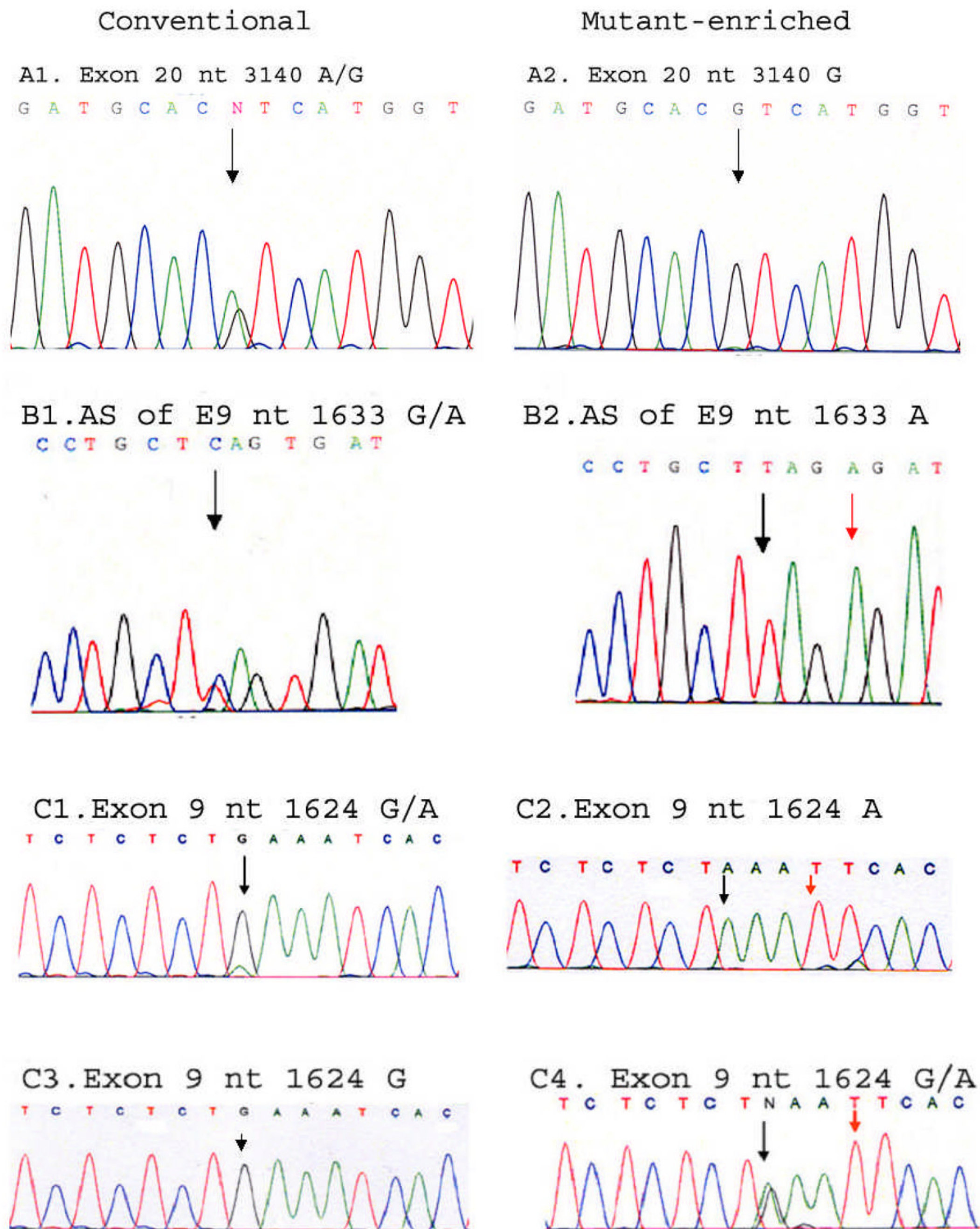

Fig. 3. The detection of *PIK3CA* hot-spot mutations by mutant-enriched sequencing

A1–2. The sensitivity of the mutant-enriched sequencing protocol for the exon 20 H1047R mutation was investigated in head and neck cell line Detroit 562, whose genome had been reported to harbor a H1047R mutation [11]. *A1.* Both wild-type A and mutant G peaks were detected at 1:1 ratio as expected by conventional genomic sequencing of the cell line Detroit 562 DNA (non-diluted). *A2.* When the ratio of mutant and wild-type DNA reached 1: 360, the mutant G peak was still the only peak detected in the cell line Detroit 562 DNA by mutant-enriched sequencing. *B1–2.* A patient sample with a known E545K mutation was used to test the mutant-enriched sequencing protocol for the exon 9 E545K mutation. *B1.* Antisense sequencing of the mutated site by the conventional genomic sequencing (marked by the black

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

arrow) detected both the wild-type and the mutant alleles. *B2*. The peak representing the wild-type allele in the same sample disappeared when the mutant-enriched sequencing method was applied (indicated by the black arrow). The red arrow marks the nucleotide A1630T change that was introduced by mismatch primer (PIK-E9MF) in order to generate the restriction enzyme Hpy188I (TCNGA) site. *C1–4*. The mutant-enriched sequencing method identified an undetected *PIK3CA* hotspot mutation E542K (G1624A) by the conventional sequencing. *C1*. Forward sequencing of a clinical sample with a known E542K mutation by the conventional genomic sequencing method (marked by the black arrow) displayed a dominant presence of the wild-type allele over the mutant allele. *C2*. The wild-type allele in the same sample disappeared when the mutant-enriched sequencing method was applied (indicated by the black arrow). The red arrow marks the nucleotide A1627T change that was introduced by mismatch primer (PIK-2E9MF) to generate a unique restriction enzyme EcoRI (GAATTC) site. The case of pharyngeal cancer that had been tested negative for a mutation by the conventional genomic sequencing method (*C3*), but was subsequently identified with a *PIK3CA* E542K mutation using the mutant-enriched sequencing method (*C4*).

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Table 1

The clinical features of five pharyngeal cancers with *PIK3CA* mutation.

No.	Gender	Age (year)	History	Histology (differentiated)	Mutation
1	Male	59	Not Available	Poorly	A3127G
2	Female	70	No smoking, occasional alcohol	Well	A3140G
3	Male	66	No smoking, occasional alcohol	Well	A3140G
4	Male	43	Not Available	Moderate	G1633A
5	Male	64	Heavy smoking, occasional alcohol	Moderate	G1624A