Oncogenic mutations of the *PIK3CA* gene in head and neck squamous cell carcinomas

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Abstract. Phosphatidylinositol 3-kinases (PI3Ks) are heterodimeric lipid kinases that regulate cellular activities such as proliferation, survival, motility and morphology. Recent studies reported that the p110α (*PIK3CA*), catalytic subunit of PI3-kinase is somatically mutated in human cancers. Hotspot mutations (E542K, E545K and H1047R) are reported to have higher oncogenic potential. Although *PIK3CA* mutations were reported in head and neck squamous cell carcinomas (HNSCC) of limited ethnicity, the functional consequences of HNSCC-associated *PIK3CA* mutations have not been examined. Status of PI3K signaling related genes (*PTEN-RAS-EGFR*) in the presence of *PIK3CA* mutation have not been reported. In this study, we analyzed exons 9 and 20 of *PIK3CA* in 54 samples, including 17 HNSCC cell lines, 19 Indian and 18 Vietnamese primary tumors. We found mutations in 29.4% (5/17) of HNSCC cell lines, 10.5% (2/19) of Indian tumors and no mutation (0/18) in Vietnamese tumors. Two homozygous *PIK3CA* mutations were found in cell lines and a novel insertion mutation with oncogenicity in Indian tumor. Analysis of PI3K signaling related genes showed that *PIK3CA* and *PTEN* mutations were mutually exclusive, though *PTEN* mutation is uncommon in HNSCC. However, *PIK3CA* mutation coexisted with *H-RAS* mutation. Furthermore, *PIK3CA* mutations were mutually exclusive to *EGFR* amplification. All the 5 mutants that we found in HNSCC, showed increased PI3 kinase activities, followed by growth factor independent higher colony forming efficiency, changes in morphology, higher rates of migration and invasion compared with *PIK3CA* wild-type. Our study is the first to examine the oncogenic potential of *PIK3CA* mutants associated with HNSCC and report on *PIK3CA* mutations in Indian and Vietnamese ethnicity. These results suggest that *PIK3CA* mutations in HNSCC are likely to be oncogenic and may significantly contribute to HNSCC carcinogenesis and pave attractive target for therapeutic prevention.

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Introduction

The phosphatidylinositol 3 kinase is a heterodimeric lipid kinase that is composed of a regulatory subunit, p85 and a catalytic subunit p110α (PIK3CA) and plays a pivotal role in regulation of various cellular signaling pathways which control cell proliferation, survival, growth, motility, cell adhesion, differentiation, cytoskeletel rearrangement and apoptosis (1,2).

PI3Ks are a large and complex family that contains three classes with multiple subunits and isoforms. The class I PI3Ks consist of two subgroups, IA and IB. The class IA can be activated by being recruited to the cell membrane via growth factor receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR) and the insulin receptor (3,4). Growth factors activate receptor tyrosine kinases (RTKs), which then activate two key signal-transduction components including the small GTPase Ras and the lipid kinase PI(3)K. Active Ras could also directly activates PI(3)K (5,6). The activated PI3K converts phosphatidylinositol-4-5-biphosphate [PI(4,5)P2] to phosphatidylinositol-3,4,5- triphosphate [PI(3,4,5)P3]. The tumor suppressor PTEN antagonizes PI3K activity by dephosphorylating PI(3,4,5)P3 to [PI(4,5)P2] (2). Accumulation of the PI(3,4,5)P3 at the plasma membrane pool brings plecstrin-homology (PH) domain-containing proteins including protein serine-threonine kinases AKT (PKB) and phosphatidylinositol-dependent protein kinase1 and 2 (PDK 1 and 2) into proximity and facilitates phosphorylation of AKT by PDK1. This phosphorylation stimulates the catalytic

activity of AKT, leads to phosphorylation of its effector proteins that affects cell growth, cell cycle entry, and cell survival (7).

Phosphatidylinositol signaling has been shown to be deregulated in various cancers. Somatic mutations of the gene encoding p85 regulatory subunit of PI3K have been reported in human colon and ovarian tumors (8). The PIK3CA, which codes for the p110α catalytic subunit of PI3K has been reported to be amplified in human cancers including head and neck squamous cell carcinomas (9). Mutations in the PIK3CA have been reported in over 25% in colorectal, gastric, breast and certain brain cancers (10), and are mutated at significant proportions in other cancer types including head and neck cancer (11-14). The *RAS* mutation and the *PTEN* inactivation by somatic mutations or loss of expression results in hyperactivity of PI3K have been reported in a variety of human cancers (6,15).

Several *PIK3CA* hot spot mutations were reported mainly in exons 9 and 20. Functional analysis of hot spot mutations such as E542K, E545K and H1047R showed increased lipid kinase activity and induced transformation in chicken embryo fibroblast (16). Further, studies on E545K and H1047R mutations by targeted homologous integration suggested that *PIK3CA* mutations are activating mutations with gain of function in tumor cell proliferation as well as in invasion and metastasis (17). These two mutations were thus evaluated to be oncogenic in transforming mammary epithelial cells (18). In addition to these mutations, few mutations in p85 binding domain (R38H), C2 domain (C420R, E453Q) and kinase domain (M1043I) were shown to increase lipid kinase activity and transform the mouse fibroblast cells (19).

Recently, mutations of *PIK3CA* were reported in HNSCC with limited ethnicity (14,32). However, no functional consequences of *PIK3CA* mutations and status of PI3K signaling related genes in presence of *PIK3CA* mutation have ever been examined in HNSCC. Further, the incidence of *PIK3CA* mutations from the most prevalent ethnicity of HNSCC such as India and Vietnam is unknown. In our present study, we analyzed *PIK3CA* gene for mutations in HNSCCs including 17 cell lines established from Japanese tumors, 19 primary tumors from India and 18 from Vietnam. We also screened for *PTEN* and *RAS* gene mutations in all the cell lines and also in tumors identified as positive for *PIK3CA* mutation with control. We constructed *PIK3CA* mutant expression vectors to study their functions. We report for the first time that the mutated *PIK3CA* genes identified from head and neck squamous cell carcinomas act as an oncogene.

Materials and methods

Cell lines. Nineteen cell lines consisting 17 head and neck squamous cell carcinoma (HNSCC) cell lines (ZA, HOC313, HOC815, NU, HOC605, HOC719, HOC927, CA922, NA, HSC2, HSC3, HSC4, HSC5, HSC6, TSU, OM1, NU), 1 cervical carcinoma (SIHA) and 1 normal embryonic lung fibroblast cell line (TIG7) were used. HNSCC cell lines were established at the first and second Departments of Oral and Maxillofacial Surgery, Faculty of Dentistry, Tokyo Medical and Dental University (20). All the cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS and 10 μg/ml gentamycin sulfate (Sigma-Aldrich, St. Louis, MO). Cells were incubated at 37˚C with 5% CO_2/95% air in a humidified CO_2 incubator until cells were lysed for genomic DNA extraction.

DNA extraction. Genomic DNA was prepared as described previously (20). Tumor DNAs prepared as previously described (21), were used in this study for Indian HNSCC. Vietnamese samples were collected by the approval of insititutional review committee at Cancer Hospital in Ho Chi Minh City, the same group of DNA samples were used for p53 mutational analysis (Hong T.N., Ph.D Dessertation, 2006).

Mutation analysis of PIK3CA, PTEN and H-RAS. The primer sequences, and the reaction conditions for the amplification of exons 9 and 20 of the *PIK3CA* gene, were as described previously (10). The full length *PTEN* gene in HNSCC cell lines was analyzed by sequencing the RT-PCR products. Briefly cDNAs were synthesized by using SuperScript™ III CellsDirect cDNA Synthesis system (Invitrogen Life Technologies, Carlsbad, CA) and the *PTEN* entire coding region was amplified as three overlapping fragments (PT1, PT2 and PT3) by using the following PCR primer sets: PT1F-CTCCTCCTTTTTCTTCAGCC, PT1R-GTCTCT GGTCCTTACTTCC; PT2F-GGGACGAACTGGTGTA ATG, PT2R-TTCCTCTGGTCCTGGTATG; and PT3F-AGC CGTTACCTGTGTGTG, PT3R-CATGGTGTTTTATCC CTC. The conditions for the PCR amplification were as follows: i) after initial denaturation at 94˚C for 1 min, a 35 cycle reaction consisting of 94˚C/30 sec, 57˚C/30 sec and 72˚C/30 sec for PT1 primer set; ii) 94˚C/30 sec, 56˚C/30 sec, and 72˚C/30 sec, for PT2 primer set; iii) 94˚C/30 sec, 54˚C/30 sec, and 72˚C/30 sec for PT3 primer set. The *PTEN* gene (exons 1-9) in HNSCC primary tumor samples (Indian and Vietnamese) was amplified using the primer sequences as described previously (22). In addition, exon 8 was amplified by using E8S-TACATTCTTCATACCAGG, and E8AS-AAGTATCGGTTGGCTTTG primers.

The *H-ras* (exons 1 and 2) was amplified using the forward primers H1S and H2S for exon 1 and 2, respectively, as described previously (23). One set of a new primer (hras1f-AGACCCTGTAGGAGGACC; hras1r-GAGGAA GCAGGAGACAGG) was used for amplifying the exon 1. Two additional primers HRAS-E1-R-CTCGCCCGCAG CAGCTGCTG, and HRAS-E2-R-GGGCCAGCCTCACG GGGTTC were used as reverse primers for exons 1 and 2, respectively. The PCR products were gel-purified as described previously (21) and sequenced by a Big Dye Terminator v1.1 cycle sequencing kit using an ABI-Prism 3100 Genetic Analyzer (Applied Biosystems).

Construction of PIK3CA expression vector. A mammalian expression vector of *PIK3CA* cDNA (EX-A1195-M01) encoding the wild-type protein was purchased from Gene-Copoeia (Frederick, MD). It was digested with *Xmn*I/*Xho*I and the resulting fragment was inserted at the *Eco*RV/*Xho*I sites of pcDNA3.1 (-) (Invitrogen). The full-length cDNA

obtained from this plasmid by partial digestion with *Bam*HI/*Xba*I was inserted at *Bgl*II and *Xba*I sites of p3XFLAG-CMV-10 (E4901) (PIK3CA/E4901). Both the 5' 1.707 kb *Xmn*I/*Xba*I fragment from EX-A1195-M01 and the 3' 1.5 kb *Xba*I fragment from PIK3CA/E4901 were ligated to the *Eco*RV and *Xba*I sites of p3XFLAG-CMV-10 (E4401) to make N-terminal FLAG fusion protein. In each step insertions were checked by restriction enzyme analyses and sequencing.

Site-directed mutagenesis. Using N-FLAG-tag PIK3CA expression vector, the *PIK3CA* mutants, E545G, E545K, M1043V, H1047R and one base insertion mutant (3191_2inA) were generated with a Quick Change XL Site-Directed Mutagenesis kit (Stratagene) and the primers, which were designed using template specific mutagenic primer design program (http://labtools.stratagene.com/QC). The primer sequences were as follows: E545G: sense, CTCTCTCTGA AATCACTGGGCAGGAGAAAGATTTTCTATG, antisense, CATAGAAAATCTTTCTCCTGCCCAGTGA TTTCAGAGAGAG; E545K: sense, TCCTCTCTCTGA AATCACTAAGCAGGAGAAAGATTTTCTATG, antisense, CATAGAAAATCTTTCTCCTGCTTAGTG ATTTCAGAGAGAGGA; M1043V: sense, TGGAGTA TTTCATGAAACAAGTGAATGATGCACATCATGG TG, anti-sense, CACCATGATGTGCATCATTCACTTGT TTCATGAAATACTCCA; H1047R: sense, ACAAATGAA TGATGCACGTCATGGTGGCTGGACA, antisense, TGT CCAGCCACCATGACGTGCATCATTCATTTGT; 3191_2inA: sense, GGATCTTCCACACAATTAAACAAG CATGCATTGAACTGAAAGA, antisense, TCTTTCAG TTCAATGCATGCTTGTTTAATTGTGTGGAAGATCC.

All the mutations were confirmed by sequencing with primers 640F AGTTCCCAGATATGTCAGTG; 641F GG TTATAAACGAGAACGTGTG for mutation in exon 9 and exon 20 respectively. Plasmid DNAs for the transfection experiments were purified, using a Qiagen endo-free purification kit.

Transfection. HEK293T cells were transfected with empty vector, wild-type or each of mutant *PIK3CA* expression vectors using Lipofectamine™ 2000 transfection reagent as per the manufacturer's instructions (Invitrogen Life Technologies). Cells were serum starved at 24 h and harvested 48 h after transfection. Non-transfected cells were treated with EGF 50 ng/ml for 10 min. These were subjected to lysis of the cells for Western blotting, immunoprecipitation and PI3-kinase assay.

Western blotting. Western blotting was performed as described (24). Briefly, proteins (20 μg) of cell lysates were separated on 8% SDS/PAGE and transferred to PVDF membrane (Millipore Co., Bedford, MA). After the membrane was blocked with 5% skim-milk/TBS (20 mM Tris-HCl, and 0.15 M NaCl) containing 0.1% Tween-20 (TBST) for 1 h at room temperature, incubated overnight at 4˚C with anti-FLAG M2 (Sigma-Aldrich), and then 1 h with anti-p85 antibody (Upstate Cell Signaling Solutions, Lake Placid, NY) at room temperature. After washing with TBST 4 times, the blots were incubated with anti-mouse and anti-rabbit HRP linked (New England Biolabs, Beverly, MA) antibodies for 1 h at room temperature. After washing with TBST, protein bands on the membrane were detected with an enhanced chemiluminescence (ECL) reaction (New England Biolabs) by exposing to X-ray film. To check the equal loading of protein, the same blot was stripped off and reprobed with anti-ß-tubulin antibody (SC-5274, Santa Cruz Biotechnology).

PI3-kinase assay. Immuno-precipitation and *in vitro* PI3-kinase assay were done as previously described (25) with a few modifications. Briefly, cells were washed with PBS, collected by scraping, and then lysed in NP-40 lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, Complete® protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), 1 mM sodium orthovanadate). Cell lysates were centrifuged for 20 min at 10000 x g at 4˚C and the supernatant was collected. The protein (150 μg) was incubated with 10 μg of anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) and anti-p85 antibody (for EGF treated cell lysate) (catalog #06-195, Upstate Cell Signaling Solutions) for 2 h at 4˚C. Pre-washed Protein G or A-Sepharose (10 μl/sample) was then added and the incubation continued for 2 h at 4˚C. After incubation, immunoprecipitates were washed 3 times with NP-40 lysis buffer, 1 time with wash buffer (500 mM LiCl, 100 mM Tris-HCl, pH 7.5), once with H_2O and with a kinase reaction buffer 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$. The immunoprecipitates were subjected to PI3-kinase assay. Briefly, the immunocomplexes were incubated with 25 μl of kinase reaction buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 200 μg/ml phosphatidylinositol (Avanti Polar Lipids Inc., Alabama, USA) dispersed from a DMSO solution to the buffer, 30 μM ATP, and 7.4 MBq/ml γ-[^{32}P]-ATP for 10 min at room temperature. The reaction was terminated by adding 40 μl of 1N HCl and extracted with 80 μl of chloroform/methanol (1:1). The organic phase was taken and the samples were dried and dissolved in a small volume of chloroform and spotted onto a pre-treated silica gel 60 F_{254}TLC plates (Merck, Darmstadt, Germany) and the plates were developed for 2.5 h in a borate buffer system (26), dried and visualized by autoradiography using image plates and an image analyzer (BAS2000, Fuji Film Co., Tokyo, Japan). Relative PI3K activities were calculated from the intensities of PIP spots by using the BAS2000 image analysis software.

Colony forming efficiency assay. Assay for colony forming efficiency was performed using SaOS-2 cells in the presence of 10% FBS or 0.5% FBS. The cells were plated at 2x10^6 per 100 mm tissue culture plate (Iwaki Technoglass, Tokyo Japan) a day before the transfection. Cells were transfected by calcium phosphate precipitation method with 1 μg of empty vector or each of expression vectors carrying the wild-type or the mutant *PIK3CA* genes (27). Forty-eight hours after the transfection G418 (Calbiochem, San Diego CA, USA) was added to the medium at 400 μg/ml to select the drug resistant colonies. The medium was replaced periodically and 3 weeks after the selection, plates were washed twice with cold PBS, fixed using methanol for 10 min, stained with 1%

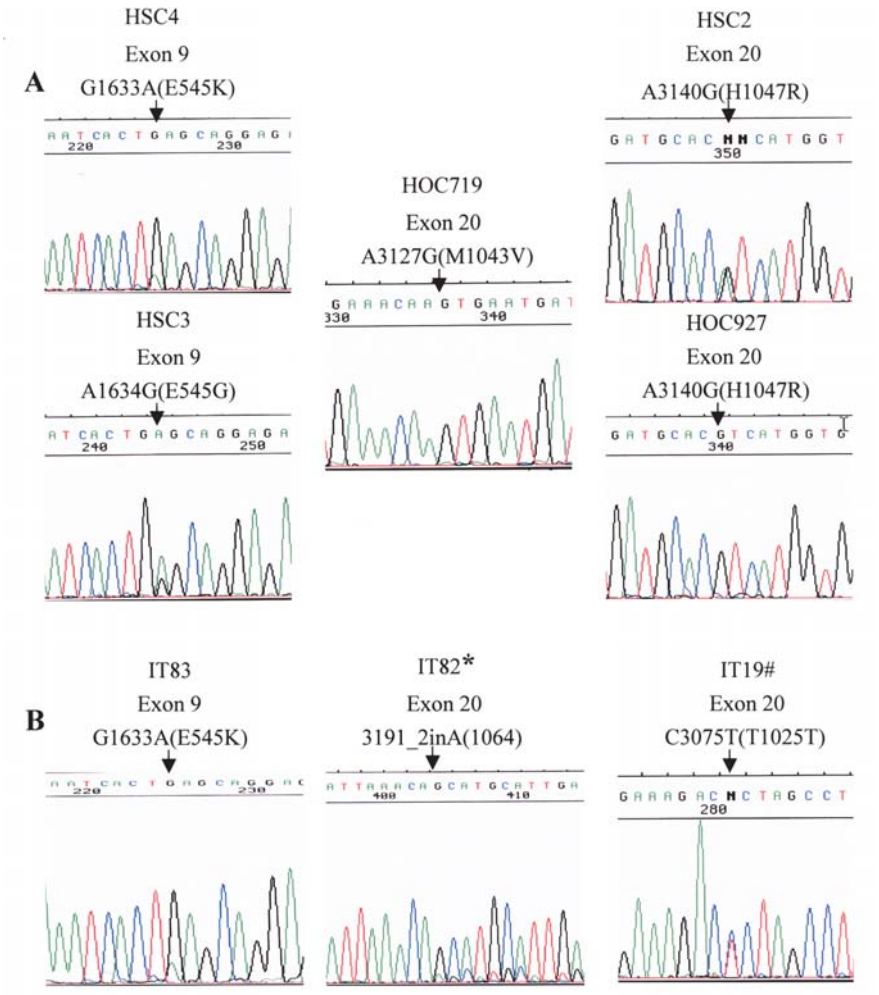

Figure 1. Sequence analysis of exons 9 and 20 of *PIK3CA* in HNSCC cell lines and Indian primary tumors. (A) Sequence profiles of exon 9 (helical domain) or 20 (kinase domain) in *PIK3CA* with somatic mutations found in HSC2, HSC3, HSC4, HOC719 and HOC927 cell lines. Arrows indicate the location of missense mutation. (B) Sequence profiles of somatic mutations in the exons 9 and 20 of *PIK3CA* and SNP (single nucleotide polymorphism) found in 9 of 19 Indian primary tumors. *, Novel mutation; #, representative example of SNP found. The nucleotide, amino acid alteration, exon and cell line name or tumor number are indicated above the arrow. Nucleotide number refers to the position within coding sequence, where position 1 corresponds to the first position of the initiation codon. All samples were sequenced in two repeated examinations with independent PCR by forward and reverse primers.

Giemsa's solution (Merck), plates were washed with tap water, followed by deionized water, air dried and the numbers of colonies were counted.

As for the observation of morphological changes, single colonies of transfected cells were focused under a microscope (Olympus BX50, Tokyo, Japan) and photographed.

Migration and invasion assay. Cell migration assays were carried out as described previously (28). Briefly, SaOS-2 cells were transiently transfected with 0.8 μg of empty vector or an expression plasmid containing the wild-type *PIK3CA* or each of mutant genes by using Lipofectamine™ 2000 transfection reagent as recommended by the manufacturer (Invitrogen). The cells were starved at 10 h of transfection. At 24 h the cells were collected and resuspended in 200 μl of serum-free DMEM with 0.2% BSA (Sigma-Aldrich). ThinCert™ (Greiner Bio-One, Longwood, FL) cell culture inserts with 8 μm pores and translucent PET membranes were placed in the wells of a CellStar™ 24-well cell culture plate and 600 μl of DMEM consisting 0.1% serum or 0.1% serum + FGF (10 ng/ml) was added to the lower compartment of each well. Cell suspension ($2x10^6$ cells) transfected with empty vector or an expression plasmid consisting wild-type *PIK3CA* or each of mutant genes was added to each cell culture inserts. The cells were incubated at 37˚C and 5% CO_2 for 22 h. The cells on the upper surface of the PET membrane were completely removed by wiping with a cotton swab, and the membranes were fixed with 70% ethanol, stained with Coomassie Brilliant Blue and the number of migrated cells per membrane was counted by microscopy. Cell invasion assays were performed using 8-μm pore-size Matrigel matrix-coated polycarbonate filters (BD BioCoat™ Matrigel™ Invasion Chamber, BD Biosciences, Bedford, MA) as described previously (17) the invaded cells were fixed and stained as explained above. The % invasion and invasion index were calculated as recommended by the manufacturer (BD Biosciences).

Results

Mutational analysis of PIK3CA gene. We analyzed the helical (exon 9) and kinase domains (exon 20) of *PIK3CA*

Table I. Summary of *PIK3CA* mutations in head and neck squamous cell carcinoma cell lines.

Name of cell line	Exon	Nucleotide no.	Codon change	Amino acid change	Type of mutation	Status of mutation	Functional domain	*PTEN* (Exl-9)	*H-RAS* (Exl&2)
HSC4	9	1633	GAG-AAG	E545K	Missense	Heterozygous	Helical	-	-
HSC3	9	1634	GAG-GGG	E545G	Missense	Heterozygous	Helical	-	-
HOC719	20	3128	ATG-GTG	M1043V	Missense	Homozygous	Kinase	-	-
HOC927	20	3140	CAT-CGT	H1047R	Missense	Homozygous	Kinase	-	-
HSC2	20	3140	CAT-CGT	H1047R	Missense	Heterozygous	Kinase	-	-
ZA	-	-	-	-	-	-	-	-	G13R[a]
HSC6	-	-	-	-	-	-	-	-	G12V

[a]Earlier report from our laboratory: Tadokoro *et al* (27). -, no mutation.

Table II. Summary of *PIK3CA* mutations in head and neck squamous cell carcinoma from India.

Tumor no.	Exon	Nucleotide no.	Codon change	Amino acid change	Type of mutation	Status of mutation	Functional domain	*PTEN* (El-9)	*H-RAS* (E1&2)
IT83	9	1633	GAG-AAG	E545K	Missense	Heterozygous	Helical	-	-
IT82	20	3191_2inA		1064	Frameshift		Kinase	-	G13R
IT19	20	3075	ACC-ACT	T1025T	Silent	Heterozygous	Kinase	-	G12V[a]
1T21	20	3075	ACC-ACT	T1025T	Silent	Heterozygous	Kinase	ND	ND
IT23	20	3075	ACC-ACT	T1025T	Silent	Heterozygous	Kinase	ND	ND
IT27	20	3075	ACC-ACT	T1025T	Silent	Heterozygous	Kinase	ND	ND
IT29	20	3075	ACC-ACT	T1025T	Silent	Heterozygous	Kinase	ND	ND
IT81	20	3075	ACC-ACT	T1025T	Silent	Heterozygous	Kinase	ND	ND
IT88	20	3075	ACC-ACT	T1025T	Silent	Heterozygous	Kinase	ND	ND
IT89	20	3075	ACC-ACT	T1025T	Silent	Heterozygous	Kinase	ND	ND
IT90	20	3075	ACC-ACT	T1025T	Silent	Heterozygous	Kinase	ND	ND
IT91	-	-	-	-	-	-	-	-	G12V[a]

[a]Earlier report from our laboratory: Munirajan *et al* (23). -, no mutation; ND, not done.

gene for mutations in HNSCC, and restricted our mutational analysis to this region as majority (75%) of the reported mutations has been found to cluster in these regions (10). In total 56 samples including 17 HNSCC cell lines established in Japan, 19 Indian tumor samples, and 18 Vietnamese tumor samples, in addition to SiHa and TIG-7 cell lines as controls were analyzed, using direct sequencing of PCR amplified products of genomic DNAs.

Out of the 17 cell lines 5 had mutations (29.4%): two mutations located at codon 545 (exon 9) one mutation at codon 1043 and two mutations at codon 1047 (exon 20). At codon 1047, two of them were identical (Fig. 1A and Table I). In two HNSCC cell lines HOC719 and HOC927, we observed loss of the wild-type allele, and thus homozygosity. We examined 19 primary tumor samples from Indian ethnic origin. All the patients were recorded to have tobacco chewing/alcohol/betel quid habits, but other clinico-pathological characteristics were not available due to poor follow-up (21). We identified 2 mutations (10.5%): one was a missense mutation located in exon 9 (codon 545), and the other was an insertion of A at codon 1064 between nucleotide 3191 and 3192 (hereafter indicated as 3191_2inA) which results in frameshift with 5 succeeding codons inclusive of stop codon. There is no information available on this mutation. As 3191_2inA is not stored in the COSMIC (Catalog of Somatic Mutations in Cancer), a database of the Sanger Institute, this is the first report of this frameshift mutation. In addition, 9 identical silent mutation/polymorphism (variants) were detected at codon 1025 in exon 20 of *PIK3CA* gene (registered in NCBI SNP database) (Fig. 1B and Table II). None of the 18 tumor samples from Vietnamese ethnic origin had a mutation in either exon 9 or 20 (data not shown).

Mutational analysis of PTEN gene. It has been reported that *PTEN* and *PIK3CA* mutations are mutually exclusive in most cases. However, in HNSCC the status of *PTEN* in the

Table III. Mutation of genes related to oncogenecity in head and neck squamous cell carcinoma cell lines.

Cell line	*PIK3CA*[a] mutation	*p53*[b] mutation	*PTEN*[a] mutation	*H-ras*[a c, d] mutation	*ERK*[e] mutation	*EGFR*[f] amplification
ZA	-	G279E	-	G13R	-	9.5
CA922	-	R248W	ND	ND	-	3.9
TSU	-	R248W	ND	ND	-	0.7
NU	-	EXON-6.s.d	ND	ND	ND	1.9
NA	-	Y220H	ND	-	-	12.3
OM1	-	G266E	ND	-	Amplified[g]	2.3
HOC313	-	E285K	ND	G12V	-	0.5
HOC605	-	Y126STOP	ND	-	-ND	2.5
HOC815	-	Y205C	ND	ND	ND	1.5
HOC719	M1043V	D281E	-	-	-ND	0.6
HOC927	H1047R	ND	-	-	-ND	0.9
HSC2	H1047R	EXON-6.s.d	-	-	-	6.2
HSC3	E545G	305 6insTAAG	-	-	-	1.1
HSC4	E545K	R258Q	-	-	-	1.7
HSC5	-	M237I	-	-	-	1.7
HSC6	-	EXON-6.s.d[i]	-	G12V	E322K	0.8
HSC7	-	ND	ND	ND	-	1.1

[a]Current study. Earlier reports from our laboratory: [b]Sakai and Tsuchida (20); [c]Sakai *et al* (42); [e]Arvind *et al* (24); [f]Matsumura (41); [g]Matsumura *et al* (43); [d]Tadokoro *et al* (27); [i]Kaneda *et al* (39). -, no mutation; ND, not done; s.d., splicing donor site.

presence of *PIK3CA* mutation is not known. We examined all the samples from *PIK3CA* mutation-positive cases and two from *PIK3CA* mutation-negative cases in each group of cell lines, and Indian tumors (Tables I and II respectively). For all Vietnamese samples we examined the mutational hot spot exons 5 and 8. We did not find any *PTEN* mutations in the cell lines and Indian tumors or in the tumor samples of Vietnamese ethnic origin.

Mutational analysis of H-RAS gene. *RAS* and its downstream effectors can activate components of PI3K-PTEN pathway through various mechanisms including mutations. We analyzed all the samples from *PIK3CA* mutation-positive cases and two from *PIK3CA* mutation-negative cases in cell lines and Indian tumors. We identified three *H-RAS* mutations: one in HSC6 cell line at codon 12, the nucleotide GGC→GTC (Tables I and III) and two mutations in Indian tumors one at codon 13 of sample IT82 and the other one codon 12 of sample IT91 changing the nucleotide GGT→CGT and GGC→GTC respectively. Interestingly, we found that the Indian tumor sample IT82 harbored concomitant *PIK3CA* and *H-RAS* mutation (Table II). We also identified two *H-RAS* silent mutations at codon 13, (G) GGT-GGG (G) in HSC2 and (G) GGT-GGC (G) in HSC6 cell lines.

Mutant p110α proteins show enhanced PI3-kinase activity. To determine whether lipid kinase activity is enhanced in the p110α mutants, *in vitro* kinase assay was performed. HEK293T cells were transfected with a control vector, or expression vectors of N-terminal FLAG-tagged wild-type and mutant PIK3CAs. The p110α complexed with p85 proteins were partially purified from the transfected cell lysates by immunoprecipitation using anti-FLAG and anti-p85 antibodies (for EGF treated sample), and assayed for the enzymatic activity using phosphatidylinositol as a substrate. As shown in Fig. 1, higher levels of PI(3)P were detected in the immunoprecipitates prepared from cells transfected with the mutant *PIK3CA* expression vectors than those transfected with the wild-type or empty-vector when cultured under the serum-free condition. All the mutant PIK3CA kinase activity levels were higher, comparable to that of EGF-stimulated HEK293T cells, which were used as a positive control (Fig. 2A and B). The immunoprecipitated protein used for PI3 kinase assay were examined further by Western blotting with anti-FLAG, anti-p85 antibodies and anti-ß-tubulin antibody as loading control. Further the mutant PIK3CA proteins were expressed at slightly higher levels than the wild-type (Fig. 2C). One of the possibilities is that mutant proteins might be relatively stable compared with the wild-type under low serum condition. The relative expression levels of mutant and the wild-type proteins were similar when cells were grown with 10% serum (data not shown).

The PIK3CA mutants enhance colony-forming efficiency of SaOS-2 cells. In order to test the effect of PIK3CA gene mutation on tumor cell growth, colony forming efficiency assay was performed in the standard serum condition (10% FBS), and low serum condition (0.5% FBS). In the standard serum condition the difference in the number of colonies between wild-type and mutant-transfected cells were not

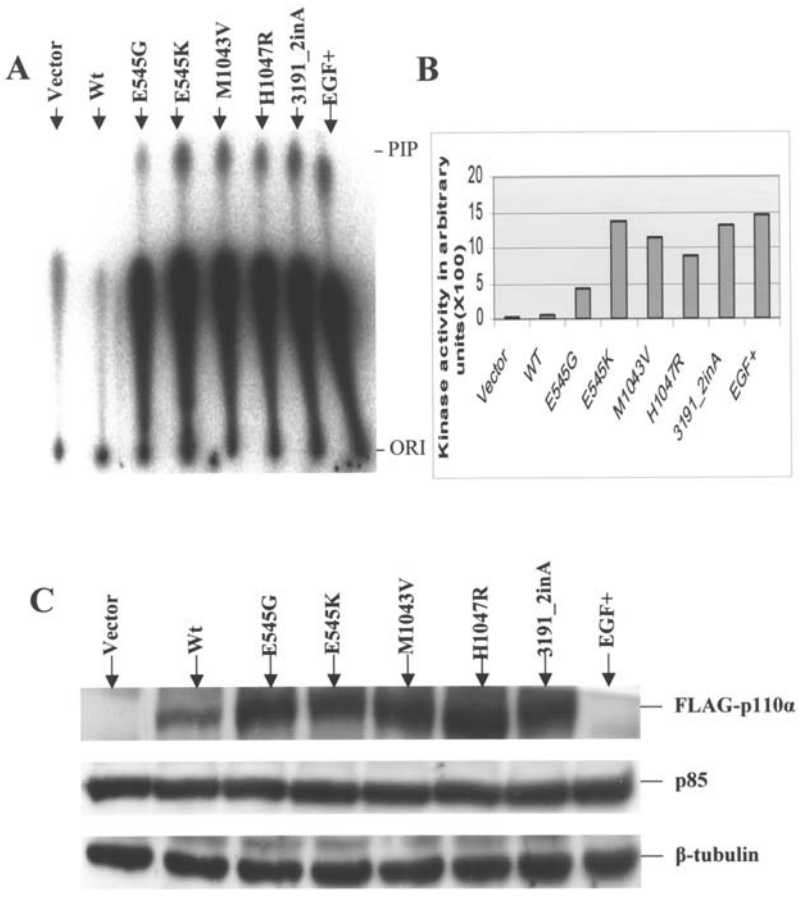

Figure 2. *PIK3CA* mutant proteins increase PI3-kinase activity *in vitro*. (A) *In vitro* PI3-kinase activity. The activities were measured with the immunoprecipitates of HEK293T cell lysates prepared after transfection with equal amount of empty vector, wt *PIK3CA*, or FLAG-tagged mutant (E545G, E545K, M1043V, H1047R, 3191_2inA) or treated with EGF (50 ng/ml) for 10 min after 24 h of serum starvation. The PI3K reaction products were separated with TLC plate, and the plate was exposed to an image plate (Bas2000, Fuji Film). PIP, phosphatidylinositol phosphate; Ori, Origin. (B) *In vitro* PI3K assays were quantified from the image film with Bas2000 Imaging & Information software and the data are plotted as arbitrary units. A representative figure of three independent experiments is shown. (C) Expression of FLAG-wt, and different mutant p110α proteins in HEK293T cells. Twenty micrograms of the indicated cell lysates used in (A) were separated on 8% SDS/PAGE and the transferred blot was probed with anti-FLAG M2, anti-p85 and anti-ß-tubulin antibodies, respectively.

significantly different (data not shown). However, in low serum condition, cells transfected with the wild-type *PIK3CA* produced less in number and smaller-sized colonies, while cells with mutants produced more in number of larger-sized colonies (Fig. 3A and B).

To determine whether *PIK3CA* gene expressed in SaOS-2 cells induces any morphological changes, G418-resistant colonies of SaOS-2 cells expressing the wild-type or each *PIK3CA* mutant was examined under a light microscope. As previously reported for NIH3T3 (19), SaOS-2 cells expressing helical domain mutants, E545G and E545K, kinase domain mutants, M1043, H1047, and 3191_2inA, cells were more refractile, less flattened and bigger in size than the wild-type cells. Each colony contained 3-4-fold more cells, which showed, increased spreading and invasive appearance when compared with those of the vector transfected and parental cells (Fig. 3C).

The PIK3CA mutant genes promote migration of SaOS-2 cells. Earlier experimental reports have shown that disruption of *PTEN* tumor suppressor gene increases the ability of the cell to migrate and the PI3K signaling was highly activated in PTEN-null tumor cell lines and primary tumors (29). We explored the effect of mutant *PIK3CA* on cell migration and invasion. It should be noted that serum concentration influences the growth of the cells expressing the wild-type p110α while that of the mutant-expressing cells is not greatly affected (17). Therefore, the migration assays were carried out in the presence of 0.1% serum concentration. FGF-treated cells served as a positive control. The migration assay showed that the cells carrying the *PIK3CA* mutant genes, E545G, E545K, M1043V, H1047R, and 3191_2inA exhibited more than 3-fold increase in their ability to migrate (as per the invasion index data) through translucent PET membrane (Fig. 4A and B) and to invade matrigel matrix-coated polycarbonate filters compared to those expressing the wild-type gene (Fig. 4C). Metastasis is an important event in cancer and migration was the primary character of a cell with oncogenecity and therefore these *PIK3CA* mutations are likely to contribute to metastasis.

Discussion

We report here the identification of mutations of the *PIK3CA* encoding catalytic subunit (p110α) of class IA PI3 kinase family. *PIK3CA* mutations were detected in 29.4% (5/17) of

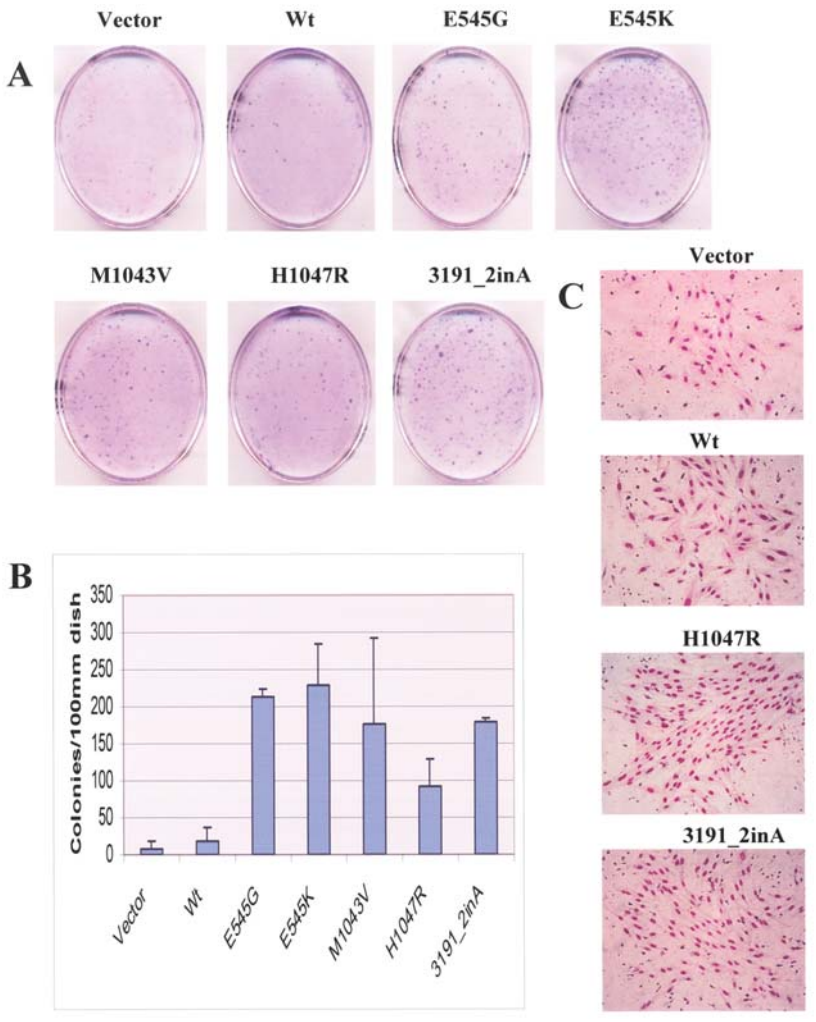

Figure 3. *PIK3CA* mutations enhance colony forming efficiency and change the morphology of SaOS-2 cells in DMEM containing 0.5%FBS. (A) Colony formation. SaOS-2 cells were transfected with vector, wt and mutants. Cells were cultured in DMEM with 0.5%FBS and selected using G418 (400 μg/ml). The colonies were stained by Giemsa's solution (1%) and counted 3-4 weeks later. (B) The number of colonies >2 mm in diameter observed after 3-4 weeks of growth was counted for each transfection. Results were indicated by the mean ± s.d. of the colony numbers from two independent experiments. (C) Morphologies of SaOS2 cells expressing *PIK3CA* wt or each of mutants. Morphology of typical colonies for each transfection are shown (vector, wt, H1047R and 3191_2insA), respectively.

HNSCC cell lines, where 2 mutations were in the codon 545 helical domain, 2 identical mutations in codon 1047 and 1 mutation in codon 1043 of the kinase domain. The mutations (E545G, E545K, M1043V and H1047R) were reported previously (10,30). Genetic aberrations that lead to a gain of functions in PI3K signaling are commonly observed in human cancers especially in solid tumors. It has been reported that PIK3CA mutations in human tumors are somatic, cancer-specific and heterozygous (5). Contrary, we found the mutations, M1043V and H1047R, in homozygous state in HOC719 and HOC927 cell lines, respectively (Fig. 1A and Table I). However, since cell lines would acquire many genetic changes during cell culture, it might happen often, even though we report for the first time. It is conceivable that tumor cells with *PIK3CA* mutation have a growth advantage and thus are more easily established as a cell line.

In Indian tumor samples, 2 mutations were found in 19 cases (10.5%); one was in codon 545 of the helical domain and the other was a novel insertion mutation, nucleotide A was inserted at codon 1064 between 3191st and 3192nd nucleotide positions (3191_2inA). In 9 out of 19 Indian samples, we found C→T nucleotide change at the position of 3075, accompanied with no amino acid change. Since this change was found in 45% of the Indian tumor samples but not in Japanese cell lines and Vietnamese tumor samples, this could be a polymorphism in Indian population (Fig.1B and Table II). The same base change was reported previously for one case of ovarian carcinoma patient in Norway (31).

We could not find any *PIK3CA* mutation in the Vietnamese tumor samples irrespective of the age group and other clinico-pathological factors, although p53 mutations were detected in 44% of the samples. One possibility for lack of mutation is that *PIK3CA* mutation might occur less frequently due to some ethnic factors, however, the mutation might be located outside the analyzed region. The mutation frequency might also depend on the selection of samples as suggested previously (11).

We found that the mutational frequency of *PIK3CA* gene at 10.5% (2/19) in Indian primary tumors (Table II) and 11% (2/17) in Japanese primary tumors (Murugan *et al*, unpublished data). Our result is comparable to that of previous *PIK3CA* mutational reports in HNSCC, 11% (4/38) in primary

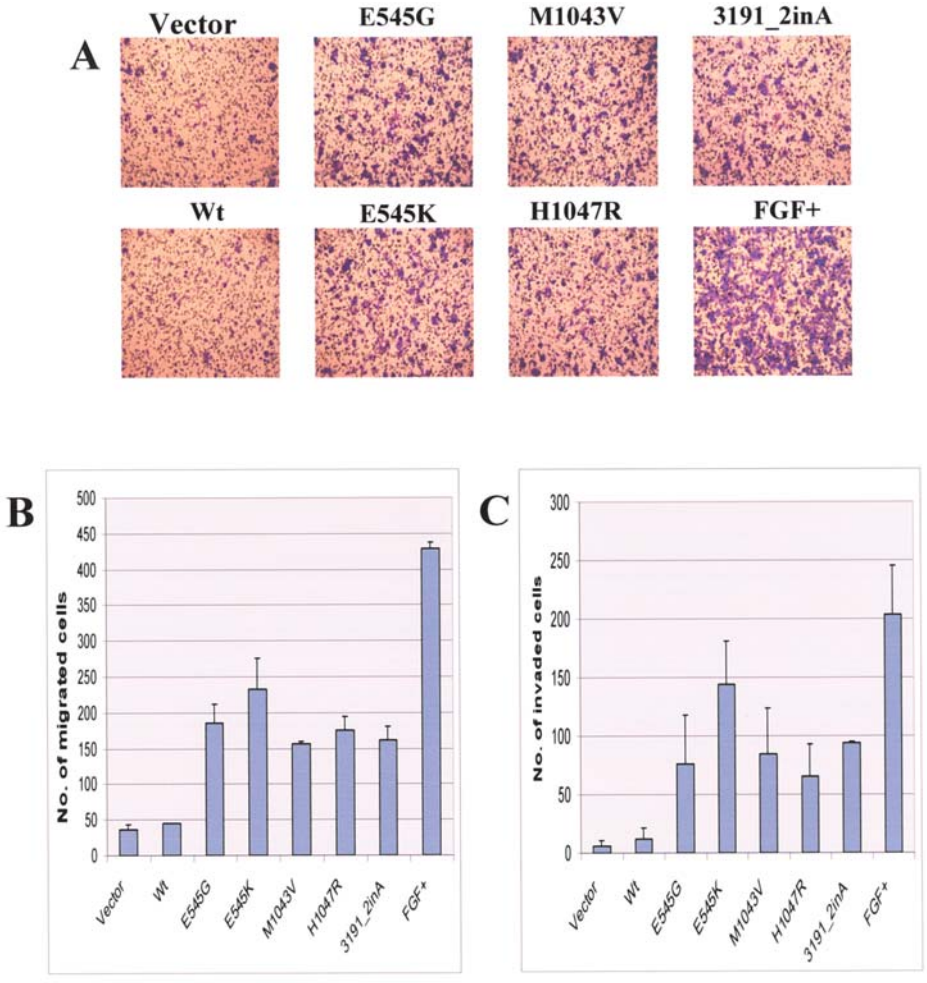

Figure 4. *PIK3CA* mutations induce migration and invasion of SaOS-2 cells. (A) *In vitro* migration assay. SaOS-2 cells were transiently transfected with equal amount of empty vector, wt or a mutant *PIK3CA* and subjected to migration assay. Migrated cells were fixed with 70% ethanol and stained by Coomassie Brilliant Blue. (B) Results in (A) are indicated by the mean ± s.d. of the migrated cell numbers from three independent experiments. (C) *In vitro* invasion assay. As described in (A), the transfected cells were subjected to invasion assay. The cells that invaded into the matrigel matrix-coated polycarbonate membrane were fixed and stained as stated above. Results obtained in invasion assay are indicated by the mean ± s.d. of the invaded cell numbers from two independent experiments.

tumors of Columbia, USA (14). While this article was in preparation, Kozaki *et al* reported that the *PIK3CA* mutations were found in 21% (3/14) of OSCC cell lines and 7.4% (total 8/108; Japanese, 2/50; Thais, 6/58) of OSCC tumors by genomic DNA sequencing (32). Moreover these findings suggest that *PIK3CA* mutation significantly contribute to HNSCC carcinogenesis. Future studies with more number of tumor samples from Indian and Vietnamese ethnicity would uncover the untold role of *PIK3CA* in HNSCC carcinogenesis.

It has been reported that *PIK3CA* and *PTEN* mutations are mutually exclusive in general. It is reasonable to postulate that increased PI3K activity via gain-of-function mutation or gene amplification might have similar effects to the loss of PTEN (11). Earlier reports are contradictory for the presence (33) and absence (34) of *PTEN* mutation in HNSCC. Interestingly, a few reports showed that *PIK3CA* mutations occur only in tumors that do not carry *PTEN* mutations (11) while in some cases of breast carcinoma, tumors have mutations both in *PIK3CA* and *PTEN* (12) and high frequency of coexistence in endometrial carcinoma (13). In our studies we could not detect any *PTEN* mutation in the cells carrying the *PIK3CA* mutations, suggesting that *PTEN* and *PIK3CA* mutations are mutually exclusive and the mutations in both genes were uncommon, if they exist (Tables I, II and III).

HNSCC in India has been reported to have relatively high incidence of *RAS* mutations (23). The *RAS* mutation is known to activate PI3K and AKT. Having significant percentage of *PIK3CA* mutation in HNSCC, raised an unresolved question whether any coexistent mutation is present in HNSCC while it has been reported in endometrial carcinoma cell line (13), colon and gastric cancer (35). We found 3 *H-RAS* mutations, all are in exon 1. One was found in the HSC6 cell line, nucleotide GGC-GTC (G12V) (Tables I and III), the other two were in Indian tumors GGT-CGT (G13R) and GGC-GTC (G12V) samples IT82 and IT91 respectively (Table II). We also found two silent mutations in HSC2 and HSC6 cell lines. Though we found 3 *H-RAS* missense mutations, only one of these samples (IT82) was positive for *PIK3CA* mutation. Our results suggest that *PIK3CA* and *H-RAS* mutations are common in significantly contributing to HNSCC carcinogenesis. The *PIK3CA* mutations may coexist with *RAS* mutation, suggesting a

possible synergistic effect in the PI3K signaling pathways controlled by these genes in HNSCC development/progression (35). However, additional studies with more number of HNSCC will help to clarify this further.

Though *PIK3CA* mutation was reported in HNSCC, functional consequences of HNSCC-associated *PIK3CA* mutation has not been examined. In this study, 5 missense mutations were analyzed for lipid kinase activity including two hot-spot mutations that were previously reported to have elevated lipid kinase activity (10,16) and we report here three new mutations: one is a hot-spot mutation (E545G) in helical domain, one non-hot-spot mutation (M1043V), and one novel insertion mutation (3191_2inA) in kinase domain. The mutations examined in this study, including the previously reported hot-spot mutations showed increased enzymatic activity when compared with the wild-type and our result is consistent with the previous reports. We observed that the mutant p110α proteins were expressed at slightly higher levels than the wild-type under low serum condition. We found that all the 5 p110α mutants produced >2-6.5-fold higher number of colonies and were larger in size, when compared to that of wild-type. Morphological changes were distinct when mutants of p110α were expressed in SaOS-2 cells and they were refractile, invasive and formed large colonies suggesting that mutant PIK3CA triggered cell growth, proliferation and survival by activation of PI3K signaling in the low concentration of growth factors and absence of EGF. Further, we examined the mutant genes for the capacity to migrate and invade *in vitro*. The newly analyzed mutations, E545G, M1043V, and 3191_2inA are able to migrate in translucent PET membrane and invade into matrigel matrix-coated polycarbonate filters, at 3- to 4-fold higher rate than the wild-ype, as were reported for the hot-spot mutation of *PIK3CA* (E545K and H1047R) (17). Therefore, we concluded that these 3 newly analyzed genes are also oncogenic, judging from *in vitro* lipid kinase activity, colony formation assay and migration and invasion assay (Figs. 2, 3 and 4 respectively). Oncogenic activities of E545G and M1043V are consistent with the report by kinase assay and focus forming assay with CEF, which was published while this manuscript was in preparation (38). The *PIK3CA*-mutated, PI3K-AKT-mediated cell proliferation and survival signaling pathways have been found to be different in each cancer type (16-18). We are presently investigating downstream effectors of AKT in HNSCC.

In HNSCC, the *PIK3CA* mutations were identified in C2 (14), helical and Kinase domains (this study). What are the molecular mechanisms by which the mutation in *PIK3CA* gene induces a gain of function? The mechanism of increased lipid kinase activity of the mutant p110α remains unclear, however it has been postulated based on the available crystal structure analyses of the related protein p110γ. It has been suggested that C2 domain in the class IB PI3K interacts primarily with the helical domain and also linker segment before the RAS-binding domain and with the COOH-terminal lobe of the catalytic domain (14), mutation causing any alteration in this function might result in activation of PI3K. In the case of helical domain Shekar *et al* demonstrated that mutations in the helical domain of p110α lead to a loss of p110 inhibition by p85 and constitutive PI3K activation (37). The kinase domain mutations including novel insertion mutation we examined have acquired gain of function since, catalytic domain mutation of p110α is analogous to the most frequently activated loop of protein kinases (38), thus it is likely that it affects specificity or affinity of p110α towards the lipid substrate of PI3K (5).

For HNSCC cell lines used in this study, we previously identified *p53* tumor suppressor gene mutations (20,39) and others reported INK4A (p16) (40). For oncogene mutations, our group reported that relatively high incidence of *EGFR* amplification (41), low frequencies of *ras* mutations (42) and *ERK2* mutations (24) and *ERK2* amplifications (43) (Table III). We are thus interested to see the relationship between *PIK3CA* and other genetic abnormalities specifically in genes of signaling proteins from EGF to ERK2 or to AKT. As is shown in Table III, there were no correlations between *PIK3CA* and *p53* mutations, both of them coexist in many of the cell lines. However, *PIK3CA* and *PTEN* mutations are mutually exclusive, since *PIK3CA*-mutated cells had no *PTEN* mutation either in cell lines or the tumors. We found only one Indian tumor with concomitant *PIK3CA* and *H-RAS* mutation. Further, the cell lines carrying *PIK3CA* mutations (4/5) had almost no amplification of *EGFR* except for HSC-2 (Table III) (39). These results are consistent with the notion that the deregulation of upstream EGFR or its effectors the PIK3CA is enough to activate the entire PI3K-AKT signaling pathway, since cell lines with amplified *EGFR* without *PIK3CA* mutation was reported to show high levels of AKT phosphorylation (32). Therefore, the presence of *PIK3CA* mutation correlated with the absence of *EGFR* amplifications in HNSCC.

In summary, our study provides additional evidence that the PI3K signaling network plays an important role in HNSCC carcinogenesis and thus is an attractive target for chemotherapy. Finding a small molecule against an oncogenic *PIK3CA* will potentially inhibit *PIK3CA* activated PI3K-AKT signaling in HNSCC.

Acknowledgements

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