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# Abnormal expression of PI3K isoforms in patients with tobacco-related oral squamous cell carcinoma

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ABSTRACT

*Background:* The phosphatidylinositol 3-kinase (PI3K) signaling regulates several cellular functions such as motility, proliferation, angiogenesis and survival.
*Methods:* Since there is no information on expression of PI3K isoforms in oral cancer, we studied the expression of different isoforms of PI3K (p110α, p110γ, PI3K-C2, Vps34p and p85α) in tumor samples and PBMC by RT and q-RTPCR and serum levels of PI3K p110α by SPR and ELISA techniques in 108 patients with tobacco-related oral squamous cell carcinoma (OSCC) and 46 normal subjects.
*Results:* We observed significantly higher PI3K p110α ($p<0.0001$) and lower ($p<0.0001$) vesicular sorting protein 34p (Vps34p) mRNA both in PBMC and tissue samples of oral cancer patients as compared to the normal controls. Other PI3K isoforms did not show such change. Circulating PI3K p110α levels were higher in patients ($p<0.0001$) as compared to healthy subjects, the SPR data showed direct correlation with advancing stage of the disease. PI3K p110α was overexpressed in tumor samples but not in the normal buccal mucosa.
*Conclusions:* Upregulation of circulating PI3K p110α isoform and its direct correlation with increasing tumor load in OSCC patients indicates that it may be a significant prognostic indicator and a suitable target for therapeutic/chemo-preventive strategies for tobacco-related OSCC.



## 1. Introduction

Oral squamous cell carcinoma (OSCC), a subgroup of head and neck malignancies ranks eighth in its global incidence. OSCC development involves accumulation of multiple genetic alterations resulting in preneoplastic lesions and eventually OSCC [1]. Despite advances in the early detection and treatment, OSCC is often diagnosed at an advanced stage that results to poor prognosis of the patient, with five-year survival rate less than 50% [2]. Thus, a better understanding of its molecular origin will contribute to improve the treatment strategies and better prognosis.

Phosphatidylinositol 3-kinase (PI3K) signaling cascade is frequently over-activated in human cancer [3–5]. Activation of PI3K localizes Akt to the plasma membrane through phosphorylation of pleckstrin homology domain of Akt at $Thr^{308}$ and $Ser^{473}$ leading to downstream signaling cascade of PI3K/Akt pathway. PI3K, ubiquitous lipid kinase, is a family of agonist stimulated lipid signaling enzymes that regulates cellular functions such as proliferation, survival, motility and angiogenesis that are critical to the growth and/or maintenance of tumors [6,7]. Three distinct classes of PI3Ks have been discovered, which differ significantly in their activation mechanisms, substrate specificity and subcellular/tissue distribution [8]. Class IA PI3K, which is mainly activated by receptor and non-receptor tyrosine kinases (RTKs), is composed of p110α, p110β or p110δ catalytic and p85, p50 or p55 adaptor subunit [9]. PI3K p110γ which belongs to class IB, is activated by G protein coupled receptor [10]. The catalytic subunits PI3K p110α (encoded by *PIK3CA*) and p110β (encoded by *PIK3CB*) are ubiquitously expressed in mammalian cells whereas p110δ being expressed predominantly in lymphocytes and lymphoid tissues may play a role in PI3K-mediated signaling in the immune system [11]. Class II isoforms are associated with the phospholipid membranes and are present in the endoplasmic reticulum and Golgi apparatus [12]. Unlike class I enzymes, these possess additional regulatory domains including the phox homology and C2 domain and lack p85/p101 binding motifs [13]. The human class III PI3K is structurally related to the yeast Vps34p [14] and mediates intracellular trafficking [13].

Over-activation of PI3K reportedly occurs in the breast, ovarian, pancreatic, esophageal, thyroid and other cancers [15]. It may be presumed that such aberrant signaling may occur either through dysfunction of pathways upstream of the PI3K class I isoforms, such as mutationally activated growth factor receptors, Ras or activation of the pathway itself or loss/inactivation of phosphatase and tensin homologue (PTEN). Amplification and mutations in *PIK3CA* gene leading to increased expression and activity of PI3K p110α and phosphorylation

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and activation of Akt have been observed in various cancers including cervical [16], lung [17], progression of dysplasia into squamous cell carcinoma [18], esophageal [19] and gastric carcinoma [20]. Since no reports are available on the status of PI3K isoforms in patients with tobacco-related OSCC, the present study was planned to evaluate the expression of different isoforms of PI3K and its correlation with clinical course of the disease.

## 2. Materials and methods

### 2.1. Patients and controls

This study included 108 oral cancer patients visiting BRA-Institute Rotary Cancer Hospital (BRA-IRCH) of All India Institute of Medical Sciences (AIIMS, New Delhi, India) and 46 age, sex and ethnicity matched healthy subjects. The healthy subjects were mostly attendant of the patients (age, 22–55 years of either gender). All patients had biopsy proven OSCC of various sites. Tumor, node, metastasis (TNM) classification and clinical staging were performed as per American Joint Committee on Cancer criteria. None of the patient had received anti-inflammatory or anti-cancer treatment prior to the study. Most of them (88%) had a history of tobacco use for >6 months. Ten milliliter of blood was aseptically collected from each individual by venipuncture; RNA and serum were separated and stored at −80 °C and −20 °C respectively until use. The study protocol was approved by the 'Ethics Committee' of AIIMS and informed consent was obtained from all participants.

### 2.2. RT and qRTPCR for expression of PI3K isoforms

The expression of different isoforms of PI3K (p110α, p110γ, PI3K-C2, Vps34p and P85α) was analyzed in the peripheral blood mononuclear cells (PBMCs) from 32 OSCC patients to 18 healthy subjects by RT-PCR while qRTPCR was performed on randomly selected 10 tumor and 10 normal tissue samples. Total RNA was extracted from peripheral blood mononuclear cells (PBMCs) and tissues (normal and tumor region) of patients using Tri reagent (Sigma Aldrich, St. Louis, MO) as per manufacturer's instructions. Tri reagent is used for convenient and simultaneous single-step isolation of DNA, RNA and proteins. It is a mixture of guanidine thiocyanate and phenol in mono-phase solution. It effectively dissolves DNA, RNA and protein on homogenization or lysis of tissues. The amount of RNA was quantified using spectrophotometer (N-1000, Nanodrop Technologies Inc., Wilmington, DE) at 260 nm absorbance and the purity of RNA was evaluated by the ratio of the absorbance at $A_{260/280}$. About 1–5 μg of total RNA was then subjected to cDNA synthesis using RevertAid H minus First Strand c-DNA Synthesis Kit (Fermentas) as per manufacturer's instructions.

cDNAs were amplified by PCR using primer specific for different PI3K isoforms as described by Krymskaya et al. [9]. Target primer sequences are shown in Table 1. PCR analyses were performed in a Thermal Cycler (Dyad Peltier PTC 220; MJ Research Inc., Incline Village, NV) using the following cycling parameters: 95 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 50 °C or 68 °C for 30 s and a final extension of 68 °C for 10 min. After amplification, an aliquot of PCR products were separated on a 1% agarose gel. Band densities were analyzed using Quantity One® image analysis software (Bio-Rad, Hercules, CA). The results were normalized against beta actin with the same RNA sample. cDNA from healthy subjects was used as a control.

Quantitative real-time polymerase chain reaction (qRTPCR) for different PI3K isoforms was performed on Bio-Rad MyIQ, Thermal cycler using SYBR Green Q-PCR Master Mix (Fermentas, Thermo Fisher, Waltham, MA). Primers were designed using the online primer designing tool, qPrimerDepot. The reaction contained 5 pmol forward and reverse primers, 100 ng cDNA, 1× SYBR Green master mix and water added to 25 μl. The primer sequences, amplicon size and annealing temperatures have been presented in Table 2. The PCR cycling parameters included initial denaturation at 95 °C for 10 min, annealing at 57 °C for 45 s and extension at 72 °C for 30 s. After amplification, a melting curve analysis was performed by collecting fluorescence data using β-actin, glyceraldehyde-3-phosphate dehydrogenase and α tubulin as internal controls. The samples were analyzed manually to obtain a threshold cycle (Cp/Ct) value [21] for each case, which was then used to determine the relative expression of each PI3K isoform using REST© software (Qiagen, Valencia, CA). All tests were performed in duplicate.

**Table 1**
PI3K isoform primer pairs, product size and annealing temperatures for RT-PCR.

| Gene name | Primer sequence 5′–3′ | Product size | Annealing temperature |
|---|---|---|---|
| *p110α* | F-CTGTGTGGGACTTATTGAGGTGGTGC<br>R-GGCATGCTGTCGAATAGCTAGATAAGC | 452 bp | 50 °C |
| *p110β* | F-GAAGATTGCAAGCAGTGATAGTGC<br>R-CCTATCCTCCGATTACCAAGTCGTC | 432 bp | 52 °C |
| *p110γ* | F-GCTTGAAAACCTGCAGAATTCTCAAC<br>R-CGTCTTTCACAATCTCGATCATTCC | 346 bp | 50 °C |
| *PI3KC2* | F-GCAGGTTCCTTCAGTCCTACTCCAGGC<br>R-GCCCAGTCAGCTGATACCATTTAACCG | 381 bp | 50 °C |
| *Vps34p* | F-CGGATCCAAAGCCTCTTCCTCCACC<br>R-CCAGTACTGGGCAAACTTGTGAATCTG | 353 bp | 50 °C |
| *β-actin* | F-AGAAAATCTGGCACCACACC<br>R-TAGCACAGCCTGGATAGCAA | 180 bp | 58 °C |

### 2.3. Serum levels of PI3K p110α by SPR

The serum levels of PI3K p110α were measured by the biosensor-based SPR technique using SensiQ Pioneer (ICx Technologies, Arlington, VA) equipment as described earlier [21]. Briefly, in the first step, preparation of carboxylated (COOH1) sensor chip (ICx Nomadics) and immobilization of the anti-PI3K p110α monoclonal antibody (BD Biosciences, San Jose CA) was performed by amine coupling (Fig. 1A). The anti-PI3K p110α antibody (100 μg/ml in 10 mmol/l acetate buffer, pH 4.5) was injected followed by 1 M ethanolamine–HCl, pH 8.5, injection for 4 min at a flow rate of 25 μl/min. Next, 5 different concentrations of recombinant PI3K p110α standard (Enzo® Life Sciences, Farmingdale, NY) in HBS-EP buffer containing 10 mmol/l HEPES (pH 7.4), 150 mmol/l NaCl, 3.4 mmol/l EDTA and 0.005% P20 (surfactant) were passed over the immobilized antibody and the corresponding resonance unit (RU) was recorded. A standard curve was plotted with RU against the concentrations (0.312, 0.625, 1.25, 2.5 and 5 μg/ml) of recombinant PI3K p110α (Fig. 1B). Serum samples diluted

**Table 2**
PI3K isoform primer pairs, product size and annealing temperatures for q-RTPCR.

| Gene name | Primer sequence 5′–3′ | Product size | Annealing temperature |
|---|---|---|---|
| *p110α* | F-GGAGCCTGGAAGAGCCC<br>R-CGTGGAGGCATTGTTCTGAT | 109 bp | 57 °C |
| *p110γ* | F-AGACAAGCCCACACTTCCAG<br>R-TTAACTGGGGCATTCCTGTC | 126 bp | 57 °C |
| *PI3K-C2* | F-CCCAGGGCCATATATCTCAG<br>R-AAAAAGCTGCCATCTCCTCA | 108 bp | 57 °C |
| *Vps34p* | F-GCAGATGGATCAGAACCCAC<br>R-TTCACCATGTGTCCTTGTCG | 116 bp | 57 °C |
| *P85α* | F-TTCATACCGTTGTTGGCTACAG<br>R-TGTGGCACAGACTTGATGTTT | 95 bp | 57 °C |
| *GAPDH* | F-GAAGGTGAAGGTCGGAGTCA<br>R-TTGAGGTCAATGAAGGGGT | 117 bp | 57 °C |
| *α-tubulin* | F-GCAACAACCTCTCCTCTTCG<br>R-CATTGCCAATCTGGACACC | 112 bp | 57 °C |
| *β-actin* | F-GCACAGAGCCTCGCCTT<br>R-GAAGGTGAAGGTCGGAGTC | 112 bp | 57 °C |