Frequent genetic and biochemical alterations of the PI 3-K/AKT/PTEN pathway in head and neck squamous cell carcinoma

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We investigated the status of the PI 3-kinase/AKT/PTEN signaling pathway in a series of 117 head and neck squamous cell carcinomas (HNSCC) in a search for molecular alterations in genes/proteins with potential prognostic value. For this purpose, PIK3CA and AKT2 gene amplification was assessed by multiplex and Quantitative Real-Time PCR. Protein expression of AKT, p-AKT, p110α and PTEN was determined by Western blot. PTEN allelic loss was evaluated by microsatellite analysis. PTEN-exon 5 was screened for point mutations by PCR-SSCP. Homozygous deletions were determined by multiplex PCR. PIK3CA gene was amplified in 43/117 (37%) fresh tumor samples, a frequency that did not differ from that found in archival premalignant tissues: 15/58 (39%); 12/40 (30%) fresh tumors harbored AKT2 gene amplification. AKT was found activated in 6/36 (17%) fresh tumor samples, when compared to their normal tissue counterparts. Of these 6 cases, 1 showed p110α overexpression and 5 displayed PTEN protein downregulation. Neither allelic loss (found in 11/77 informative cases) nor point mutations or homozygous deletions accounted for the reduced PTEN protein expression observed in our tumor series. The histologically normal mucosa of 4 patients displayed some of the molecular alterations analyzed. Dysregulation of the PI 3-K/AKT/PTEN pathway might contribute to certain HNSCC tumorigenesis and might constitute a potential clinical target. Overall, 17/36 (47%) cases showed at least 1 of the molecular alterations studied here, which makes the PI 3-kinase-initiated signaling pathway one of the most frequently altered in HNSCC.

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Key words: Head and neck squamous cell carcinoma; PTEN; PIK3CA; AKT2

Head and Neck Squamous Cell Carcinoma (HNSCC) is one of the most common types of tumors, afflicting 500,000 patients worldwide each year.1 HNSCC is thought to progress through a series of well-defined clinical and histopathological stages, which correlate with the accumulation of multiple genetic events. This led Califano et al.2 to propose a molecular progression model for this type of tumor.

The recognized risk factors for head and neck carcinoma (heavy smoking and high alcohol consumption) and the clinical appearance of premalignant lesions are poor predictors of risk of tumor development. Molecular markers that can be used for cancer-risk assessment are needed.

Class I phosphatidylinositol 3-kinase (PI 3-K) plays a central role in cellular proliferation, motility, neovascularization, viability and senescence. This cytosolic enzyme consists of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit (p110α) that phosphorylates inositol-containing phospholipids. One target molecule of PI 3-K and the phospholipid second messengers is the serine-threonine kinase AKT. The phosphorylated products of PI 3-K bind to AKT/PKB and cause its membrane translocation, which brings it in proximity to PDKs 1 and 2. Subsequent phosphorylation of AKT/PKB by these kinases results in its efficient activation. AKT2 is involved in oncogenic transformation by promoting survival of tumor cells under conditions that would otherwise lead to their elimination.

The recently identified tumor suppressor protein phosphatase and tensin homologue deleted from chromosome 10 mutated in multiple advanced tumors (PTEN)3,4 has been shown to dephosphorylate the PI 3-K-generated 3'-phosphorylated phosphatidylinositol-3,4,5-trisphosphates in vitro, thus interfering with the potentially oncogenic signals emanating from PI 3-K.5 Inactivating mutations or loss of PTEN expression lead to increased levels of PI 3-K products in cells. PTEN −/− is embryonically lethal and PTEN−/− mouse embryo fibroblasts exhibit markedly decreased sensitivity to apoptosis induction by various stimuli and increased AKT/PKB activity.6

Recent evidence has shown that PIK3CA at 3q26.3, which encodes the p110α catalytic subunit of PI 3-K, is an oncogene in ovarian and cervical carcinomas.8,9 Additionally, AKT2 (19q13.2) is of clinical interest due to its frequent amplification in human pancreatic, breast and ovarian tumors.10,11 Since gene amplification is one of the essential mechanisms of oncogene activation and amplification of 3q as well as overrepresentation on chromosome 19 are frequent aberrations found in head and neck squamous cell carcinoma, we sought to determine the possible contribution of PIK3CA and AKT2 amplification to HNSCC development.

Loss of heterozygosities (LOH) is associated with the presence of tumor suppressor genes. A CGH study revealed a specific association between loss of genetic material on 10q and nodal metastasis in HNSCC.12 It is unknown which genes are inactivated by 10q23 deletions in these tumors.

It is still unclear whether alterations in the PI 3-K-initiated signaling pathway play a role in HNSCC development. In our study, we sought to determine whether this contribution does exist and whether any of the molecular alterations in this pathway could serve as molecular markers with prognostic value that could be useful in clinical practice.

Abbreviations: CGH, comparative genomic hybridization; CIS, carcinoma in situ; ECL, enhanced chemiluminescence; FISH, fluorescence in situ hybridization; HNSCC, head and neck squamous cell carcinoma; IHC, immunohistochemistry; LOH, loss of heterozygosity; PCR-SSCP, polymerase chain reaction-single strand conformation polymorphism; PDK, 3-phosphoinositide-dependent kinase; PI 3-K, phosphatidylinositol 3-kinase; PI(4,5)P2, phosphoinositol 3,4-bisphosphate; PI(3,4,5)P3, phosphoinositol 3,4,5-trisphosphate; PKB, protein kinase B; PMSF, phenylmethylsulfonylfluoride; PTEN/MMAC, phosphatase and tensin homologue deleted on chromosome 10 mutated in multiple advanced tumors; RT-PCR, reverse-transcription-polymerase chain reaction; TNM, tumor-node-metastasis staging.

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Material and methods

Tissue specimens and DNA extraction.

Surgical tissue specimens from 117 patients with HNSCC, who consecutively underwent resection of their tumors at the Hospital Central de Asturias were prospectively obtained for our study, following institutional review board guidelines. Informed consent was obtained from each patient. None of them had received radio/chemotherapy prior to intervention nor were they thought to have distant metastases at the time of diagnosis. Pieces were sharply excised, placed in sterile tubes and frozen immediately in liquid nitrogen. In some cases, clinically normal contralateral or adjacent mucosa was also collected and the diagnosis was confirmed histologically by the pathologist. This tissue was considered as “normal mucosa” in the remaining patients, blood (10 ml) was obtained by venipuncture and leucocyte DNA was isolated for use as control DNA. All tissue samples were stored at −80°C until analysis. Some samples were bisected before freezing, half of which was used for DNA extraction and the other for protein extraction.

For DNA extraction, freshly frozen tissue from each sample was microdissected by cryostat sectioning to ensure it contained at least 75% epithelial tumor cells. These samples, as well as those from normal mucosa, were digested in lysis solution (10 mM Tris, pH 7.5, 50 mM NaCl, 10 mM EDTA, 1% SDS and 1 mg/ml proteinase K) at 37°C overnight, followed by phenol-chloroform extraction and ethanol precipitation. Isolation of leucocyte DNA was undertaken following the salting out procedure as previously described.13

The characteristics of the studied patients and the clinicopathological features of their tumors [site, pT category, pN category, TNM stage, according to the tumor-node-metastasis staging (TNM) system of the International Union Against Cancer, 6th Edition, histopathologic grade and R classification]14 are shown in Table I. Detailed distribution of cases throughout all TNM stages were the following: 8 patients (7%) stage I, 10 (8%) stage II, 34 (29%) stage III, 49 (42%) stage IV, 26 (22%) stage V, and 14 (12%) stage VI. For statistical analysis, these were grouped into 3 categories: I–II and III–IV. All the patients were habitual tobacco and alcohol consumers.

### Premalignant lesions

Precancerous lesions preceding HNSCC include a series of pathologically well-defined steps: hyperplasia (a nonneoplastic lesion consisting of the thickening of the epithelium usually caused by a response to injury or irritation), dysplasia (characterized by several morphologic changes, including the presence of mitoses, pleomorphism and prominent nucleoli) and carcinoma in situ (CIS) (when dysplasia involves the entire thickness of the mucosa). These lesions represents steps along a continuum from normal to malignant epithelium. According to this classification, tissue was obtained from archival, paraffin-embedded blocks from the Hospital Central de Asturias in order to perform a retrospective analysis: 16 cases showing hyperplasia, 7 cases with mild dysplasia, 10 cases with moderate dysplasia and 3 cases with severe dysplasia/CIS. Representative sections from tissue were used for DNA extraction and the diagnosis was confirmed for each lesion by a pathologist. The distribution of patients according to the histological nature of their lesions: hyperplasia, dysplasia and CIS is shown in Table II.

The microdissected samples were placed in xylene (3 × 30 min) to remove the paraffin, pelleted in ethanol, dried and incubated in lysis buffer (50 mM Tris pH 8.5, 10 mM EDTA, 0.5% Tween20, 1 mg/ml Proteinase K) at 55°C overnight. Digested tissue was subjected to phenol-chloroform extraction and ethanol precipitation.

Normal control DNA was isolated from paraffin-embedded tissue of pharyngeal mucosa obtained from nononcologic patients surgically treated of tonsillectomy.

### Gene amplification

Multiplex PCR permitted a semiquantitative assessment of PIK3CA gene amplification. Oligonucleotides were designed for the simultaneous amplification of a 150 bp fragment of PIK3CA gene (3p21), and a 187 bp segment of COL7A1 (3p21), a single copy gene taken as control. PCR reactions were performed in a final volume of 20 ml containing 200 mM deoxynucleotide triphosphates, 2.5 mM MgCl2, 200 ng DNA, 0.5 mM each oligonucleotide and 0.5 U AmpliTaq GOLD (Applied Biosystems, Foster City, CA). Reaction conditions were as follows: a 10 min step at 95°C (enzyme activation) followed by 30 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min and a final 10 min step at 72°C. PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining. The same approach was used to study AKT2 gene amplification (19q13), taking the GPR4 gene (19q13) as control. This approach was undertaken on fresh tumors and premalignant lesions, except for the AKT2 gene, which was only studied on fresh tumors. All reactions were carried out at least twice in independent experiments.

Genes chosen as controls are located on the same chromosome as the target gene to ensure that the increase in band intensity, suggestive of an increase in copy number, is not the result of the corresponding chromosome polysomy.

All oligonucleotides were purchased from Life Technologies (Bethesda, MD), and their sequences, annealing temperatures and amplified fragment size are shown in Table III.

![Table](https://via.placeholder.com/150)

**Table I.** Detailed distribution of cases throughout all TNM stages for each parameter. The characteristics of the studied patients and the clinicopathological features of their tumors [site, pT category, pN category, TNM stage, according to the tumor-node-metastasis staging (TNM) system of the International Union Against Cancer, 6th Edition, histopathologic grade and R classification]14 are shown in Table I. Detailed distribution of cases throughout all TNM stages were the following: 8 patients (7%) stage I, 10 (8%) stage II, 34 (29%) stage III, 49 (42%) stage IV, 26 (22%) stage V, and 14 (12%) stage VI. For statistical analysis, these were grouped into 3 categories: I–II and III–IV.

![Table II](https://via.placeholder.com/150)

**Table II.** Distribution of cases with PIK3CA amplification throughout the different groups of premalignant lesions. Hyperplasia: 16 (42%) 6 (37) 0.597

Mild/moderate dysplasia: 17 (45%) 6 (35)

Severe dysplasia/CIS: 5 (13%) 3 (60)

Total: 38 15 (39)
Retracted

Microsatellite LOH analysis

DNA from paired tumor and normal mucosa head and neck surgical specimens was analyzed for LOH at the chromosomal region containing the PTEN gene (10q23) by PCR amplification of dinucleotide repeat-containing sequences. The microsatellite markers used were D10S2492 (intragenic to PTEN) and D10S2491 (5’ end of the gene). Primer sequences were obtained from the Genome Database and commercially synthesized (Invitrogen, Life Technologies). PCR conditions consisted of 1 step at 95°C for 10 min followed by 32 cycles at 95°C for 30 sec, Tm for 1 min (58°C or 62°C for D10S2492 or D10S2491, respectively), at 72°C for 30 sec and a final single step of 72°C for 10 min. Six microliters of the PCR products were mixed with 3 μl of denaturing loading buffer 3× (95% formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue), heated at 98°C for 5 min and electrophoresed on 10% polyacrylamide-urea-formamide gels (19:1), which were then fixed in 10% acetic acid, silver stained in a freshly prepared silver nitrate (0.1%) and developed in sodium carbonate (3%) with formaldehyde [0.05% (v/v)] and sodium thiosulphate (0.2%).

For informative cases (heterozygotes), allelic loss was recorded if the intensity of the signal from one allele was reduced at least 50% in the tumor DNA when compared to that in the normal DNA on visual inspection by 2 independent observers (JMGP and MGV).

Homozygous deletion assessment of PTEN

PTEN-exon 5 (which codes for the phosphatase domain) was studied in a search for homozygous deletion on tumor specimens. Multiplex PCR was performed under 24 cycles, using a pair of primers spanning the entire sequence of exon 5 (5A-Fwd and 5B-Rvs) (309 bp) and taking GPX4 gene as amplification control (174 bp) (Table III). PCR products were electrophoresed on non-denaturing 12% polyacrylamide gels (29:1) and visualized by silver staining as described above.

PCR-single strand conformation polymorphism (PCR-SSCP) analysis of PTEN

Those paired normal/tumor samples that showed LOH for at least 1 of the markers analyzed were subjected to PCR amplification of PTEN-exon 5 split into 3 fragments of 185 and 177 bp (primer pairs 5A and 5B, Table III). PCR-SSCP analysis was adapted from the original method of Orita et al.16 Briefly, PCR products were separated on nondenaturing 12% polyacrylamide gels (29:1) containing 10% glycerol and silver stained as described above.

Protein extraction and Western blotting

Each sample was frozen and thawed 3 times and mechanically lysed in ice-cold lysis buffer containing 50 mM HEPES, pH 7.9, 250 mM NaCl, 5 mM EDTA, 0.2% (v/v) NP-40, 10% glycerol plus a phosphatase- and protease-inhibitor mixture [PPIM: 25 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml leupeptin and 10 μg/ml aprotime]. Whole protein extract concentration of the supernatant was estimated by Bradford’s method using a protein assay kit (Bio-Rad, Richmond, CA). Equal amounts of protein extract (200 μg per lane) were boiled in Laemmli sample buffer and separated on SDS/polyacrylamide gel (10%) and transferred to PVDF membranes using Mini Trans Blot Transfer Cell (Bio-Rad). Membranes were immunoblotted with rabbit polyclonal phospho-AKT-Ser-473 antibody (1:1,000 dilution), and rabbit polyclonal AKT antibody (56 kDa) (1:1,000 dilution) (New England Biolabs, Beverly, MA).
MA), goat polyclonal PI 3-K (p110α) (sc-1332) (1:500 dilution) and goat polyclonal PTEN (sc-6818) (1:250 dilution) antibodies. Anti-goat or anti-rabbit IgG secondary antibodies were used at a 1:2,000 dilution. For protein load control, anti β-actin mouse monoclonal antibody (Sigma Chemical Co., St. Louis, MO) was used. Anti-mouse IgG secondary antibody was used at 1:25,000 dilution. Immunoreactive bands were visualized using the enhanced chemiluminescence Western blotting analysis system (ECL, Amersham-Pharmacia-Biotech, Arlington Heights, IL).

Statistical analysis

The molecular results data distributed among the different clinical groups of tumors were tested for significance employing the chi-square test with the help of the statistical software package SPSS (SPSS, Inc., Chicago, IL). p < 0.05 values were considered statistically significant.

Results

PIK3CA and AKT2 gene amplification: clinical correlations

We employed a semiquantitative approach to determine the relative number of copies of the PIK3CA gene (3q26) present in tumor/premalignant lesions with respect to their normal tissue counterparts. Multiplex PCR was performed, in which a single copy gene was taken as control for signal intensity corresponding to one copy. Oligonucleotide pair proportions were adjusted to obtain the same yield for both targets on normal samples. The results obtained under these experimental conditions on tumor/premalignant lesions were considered and analyzed. Figure 1a shows an example of how the presence of increasing amounts of a fragment of the PIK3CA gene (previously PCR amplified and purified) is capable of competing for PCR amplification to the extent of being the only amplified target. Of the 117 fresh tumors studied, 43 (37%) exhibited a signal intensity that suggested the presence of more than one copy of the PIK3CA gene (Fig. 1a). No statistical significance was found for the correlation between PIK3CA amplification and any of the clinicopathological parameters shown in Table I. Additionally, 38 premalignant lesions were studied for PIK3CA gene amplification by the same method. 15 of these (39%) were positive for this alteration and were equally distributed over the different subtypes of premalignant lesions (Table II).

We validated this semiquantitative approach by comparison to Q-PCR. The same results were obtained by both methods. Quantitative data for some cases are shown as representative examples

### Table IV – Gene amplification analysis of PIK3CA by Q-PCR

| Sample | Normalized PIK3CA | PIK3CA gene amplification |
|--------|-------------------|---------------------------|
| T19    | 1.0433            | 3.67                      | Positive |
| N19    | 0.2843            |                           |          |
| T22    | 1.2832            | 2.986                     | Positive |
| N22    | 0.4297            |                           |          |
| T126   | 0.3995            | 1.138                     | Negative |
| N126   | 0.3509            |                           |          |
| T5     | 0.3714            | 0.6275                    | Negative |
| N5     | 0.5919            |                           |          |

1Data listed are for representative examples of those analyzed by Q-PCR (SYBR Green). 2Values provided represent means derived from triplicate assays of the same sample. Mean amplification values of PIK3CA divided by the mean amplification values of COL7A1 obtained from corresponding standard curves. 3PIK3CA values normalized for each tumor sample (T) to its normal tissue (N) counterpart.

We also analyzed AKT2 gene amplification following the semiquantitative approach. In this case, 12 out of 40 (30%) tumors studied showed an intensity signal suggestive of the presence of more than one copy gene. We also found this genetic alteration in the histologically normal mucosa of 2 patients (Fig. 1b). No correlation with clinical parameters was found.

Activation of AKT/PKB protein

Western blot analysis of total AKT/PKB and its phosphorylated form (p-AKT) on protein extracts obtained from normal and tumor tissue allowed the assessment of the activation state of this protein in our series. Protein extracts were available for 36 cases of which 6 tumors (17%) and 4 histologically normal mucosas displayed a stronger intensity band for p-AKT. All samples, including those harboring AKT2 gene amplification showed the same total AKT band intensity (Fig. 2a).

FIGURE 1 – Gene amplification assessment of PIK3CA (a) and AKT2 (b) genes by multiplex PCR, taking COL7A1 and GPX4 as control genes, respectively. (a) Increasing amounts of a PIK3CA gene fragment present in the reaction vessel compete with the control gene for PCR amplification. Reactions were performed on DNA from leucocytes (L), normal (healthy) mucosa (N) and tumor (T) of each patient. PCR products were resolved on agarose gels (2%) and DNA was visualized by ethidium bromide staining. Case 113 shows an overrepresentation of PIK3CA and a single copy of AKT2, while case 101 displays an overrepresentation of AKT2 in both tumor and histologically normal mucosa.

FIGURE 2 – Western blot analysis of pAKT, total AKT (a), PTEN (b) and p110α (c) in normal (N) and tumoral (T) protein extracts. Case 98 shows normal levels of all proteins; tumor from patient 88 displays accumulation of pAKT and p110α, PTEN being downregulated; tumor from patient 101 harbors overexpression of p110α, while both histologically normal mucosa and tumor show pAKT accumulation and PTEN downregulation (or absence). In each case normal levels of total AKT were detected (a). Detection of β-actin was used for protein load control (d).
No statistical correlation was found between AKT activation in tumors and any clinical parameter. However, there was an association between the presence of this molecular alteration in the mucosa and the primary tumor site, this being more frequent in those patients with pharyngeal cancer \( (p = 0.018) \).

**Overexpression of p110α protein. Loss of PTEN protein expression**

Studies carried out on cell lines have shown that accumulation of p-AKT in response to growth factors is dependent on PI 3-K activation or, alternatively, on a loss of PTEN protein phosphatase function. Thus, we investigated whether p110α overexpression and/or PTEN downregulation could be responsible for the AKT activation detected in our tumor samples.

Western blot analysis was therefore performed to assess p110α and PTEN protein levels in our tumor samples, comparing these to those found in the corresponding normal tissue. We detected p110α overexpression in 1 of the 6 tumors showing pAKT accumulation (Fig. 2c) while PTEN protein was downregulated in the remaining 5 cases (Fig. 2b). We found a correlation between PTEN downregulation and pT category \( (p = 0.026) \). Abnormal p110α and PTEN expression was observed in 1 and 3 additional cases showing normal levels of pAKT, respectively. Interestingly, 3 out of the 8 patients with abnormal PTEN expression showed the same alteration in their mucosas. There was a tendency towards it being more frequent in patients with tumors arising at the pharynx, although statistical significance was not reached \( (p = 0.051) \).

**Deletion and mutation analysis of PTEN gene**

Deletion state of the PTEN gene was evaluated in order to gain further insight into the possible mechanism responsible for PTEN reduced expression detected in our HNSCC samples. First, we determined the incidence of LOH at 10q23, where PTEN maps, by comparing the microsatellite patterns of DNA samples from 83 HNSCC with those from their normal tissue (or leucocyte) counterparts (Fig. 3a). Two 10q23 microsatellite markers were used, one of which (D10S2492) is intragenic to the PTEN gene (introns 8) and the other (D10S2491) is centromeric to the gene. Thus, LOH at this region can be attributed to the loss of one of the PTEN alleles. A total of 77/83 cases (93%) were informative (heterozygotes) for at least one of the PTEN markers, and LOH was found in the tumor tissue of 11 patients (14%) (Fig. 3a). No significant differences in the rate of 10q23 LOH were found with respect to any clinicopathological parameter.

We amplified PTEN-exon 5 in the 11 cases showing LOH to elucidate the mutational status of the remaining allele. PCR-SSCP analysis showed an absence of abnormal electrophoretic patterns that would suggest the presence of a mutation in exon 5 (Fig. 3b).

The fifth exon of PTEN was amplified to detect homozygous deletions of the gene. Multiplex PCR was performed on reduced number of cycles (24, what corresponds to the exponential phase of the reaction) in order to minimize the signal produced by contaminant nontumor DNA. This approach has allowed us to detect homozygous deletions in other genes. In our study, we used GPX4 as an internal control. However, we obtained a PCR product for PTEN-exon 5 in all but one case (Fig. 3c).

**Discussion**

Much of the complex fundamental biology of HNSCC remains poorly understood, despite intensive study. This work focuses on the analysis of molecular alterations associated with the PI 3-K/AKT pathway, one of the best characterized signaling pathways that promotes cellular survival and resistance to apoptosis. The oncogenic transformation by PI 3-K depends on its constitutive lipid kinase activity and does not require binding of p110α to the regulatory subunit p85. Deletion of the entire kinase domain \( (768–1068 \text{ aa}) \) abolishes the kinase activity of p110α. Given its importance, primers directed against this domain were used to study the amplification state of the gene. Our results show that amplification of the PTEN gene is a frequent early event in HNSCC (37%). This genetic alteration was present in premalignant lesions with a frequency (39%) that did not differ significantly from that of advanced tumors. Redon et al. have recently found by a comparative genomic hybridization-fluorescence in situ hybridization (CGH-FISH) approach that partial or total 3q gain, affecting the PIK3CA oncogene, is a common event in low-grade HNSCC without lymph node involvement or distant metastases.

The results of our PCR-based approach on a much wider tumor and premalignant series support this idea.

3q21-q29 gain has been identified as an independent prognostic marker in HNSCC by CGH analysis of tumors with variable grades and stages. Although we found no statistical significance for the correlation between PIK3CA amplification and any clinicopathological parameter, a longer patient follow-up will allow us to perform a survival analysis in the near future.

Increased PIK3CA copy number has been associated with increased PIK3CA transcription, p110α protein overexpression and increased PI 3-K activity in human ovarian and cervical cancer cell lines. The fact that no correlation between p110α protein expression and PIK3CA gene amplification was found in our HNSCC series points to other mechanisms distinct from gene amplification to explain p110α overexpression detected in our study. A very attractive possibility would be what West et al. have very recently demonstrated in lung carcinoma, another tobacco-related tumor. They showed how nicotinic activation of AKT depends upon PI 3-K and specific nicotinic acetylcholine receptors (nAchRs), although the mechanism of coupling of nAchRs to the PI 3-K/AKT pathway is unknown.

The overrepresentation of chromosome 19q is a typical finding on small cell lung cancer, which is characterized by early and widespread metastasis formation. 19q gain has also been associated with poorly differentiated HNSCC. A possible candidate target gene for this alteration is AKT2 (19q13.2), which is amplified and overexpressed in 20% of pancreatic ductal adenocarcinomas and in 30% of the HNSCC series studied here. The finding of this genetic alteration in the histologically normal mu-
cosa of two patients would suggest that it represents an early event in tumor development. AKT2 might be a target of the action of carcinogens on the mucosa of the upper aerodigestive tract and could represent one of the earliest steps in the development of a second primary tumor, a frequent event in HNSCC patients.

Snaddon et al.\textsuperscript{25} have reported normal p-AKT protein levels on HNSCC cell lines, which differs from our observations on fresh tumor samples (6/36 cases). The fact that this alteration has been already observed in 4 histologically normal mucosas suggests it might play a role in HNSCC development. Supporting this idea, West et al.\textsuperscript{31} have shown that stimulation with nicotine induced phosphorylation of AKT in short-term cultures of normal human bronchial epithelial cells, through loss of contact inhibition and development of resistance to apoptosis. Activated AKT could also be detected in lung cancers (an environmental cancer commonly associated with tobacco consumption, as it is HNSCC) derived from smokers, which suggests that AKT activation is maintained throughout tumorigenesis and might possibly be necessary for tumor maintenance. This could also apply to HNSCC.

Although controversial results have been reported regarding PTEN contribution to HNSCC tumorigenesis, the data presented here indicates that AKT activation observed in our study could be the consequence of a loss or a reduction of PTEN expression in most cases (5 out of 6), whereas it could be accounted for by p110α overexpression in the remaining case. The fact that PTEN downregulation correlated with pT category (p = 0.026) points to its possible involvement in head and neck tumor formation, consistent with the proposed mechanism of action for this protein.\textsuperscript{26}

Other authors have reported that loss of PTEN protein expression is not uncommon and has been shown to be an independent predictive factor of poor outcome in tongue cancers.\textsuperscript{27}

Regarding allelic losses affecting PTEN gene, our results are in agreement with those reported by others on HNSCC cell lines and primary tumors.\textsuperscript{28} No significant differences in the rate of 10q23 LOH were found with respect to any clinicopathological feature, which differs from the specific association between loss of genetic material on 10q and nodal metastasis reported in a CGH study on HNSCC.\textsuperscript{15} Another gene could be the target of these deletions.

We amplified PTEN-exon 5 in the 11 cases showing LOH in a search for mutations in the remaining allele, since exon 5 codes for the lipid phosphate domain of PTEN, which is essential for the tumor suppressor function of this protein.\textsuperscript{26,29} Its functional importance is underscored by the fact that PTEN-exon 5 participates actively in the acquisition of transformed characteristics when mutated in a nontransformed Cowden disease cell line, and a mutational hotspot has been described for PTEN.\textsuperscript{30} Although it is possible that mutations on other exons would be missed in our study, our results are in agreement with those reported by Henderson et al.\textsuperscript{31} who found no point mutations in PTEN gene in HNSCC. On the other hand, the finding of only one case showing PTEN homozygous deletion in line with the results reported by Chen et al.\textsuperscript{32} who found no single homozygous deletion in 28 fresh oral squamous cell carcinomas. Although genetic alterations of the PTEN gene seem to be rare in HNSCC, the low protein levels detected in our study, responsible for p-AKT accumulation in the majority of the cases, suggests that it might have a contribution on the development of this type of tumor. LOH and mutations of the PTEN gene do not account for PTEN protein downregulation observed here. Thus, inactivating mechanisms other than deletions or mutations must be responsible for PTEN downregulation (such as gene silencing by promoter methylation, as has been suggested in prostate carcinoma).\textsuperscript{33} Another possibility that has been suggested for lung carcinoma\textsuperscript{21} is that nicotine could inactivate PTEN by stimulating phosphorylation of residues in its C-term, which has been previously shown to result in degradation of PTEN. Our results support the hypothesis that carcinogens promote tumorigenesis through both biochemical and genetic mechanisms. Interestingly, our finding of histologically normal mucosas that were positive for AKT2 amplification, p-AKT accumulation and PTEN decreased expression provides evidence that carcinogen-containing environment that initiated tumorigenesis also affected the nontransformed surrounding tissues, in accordance with the theory of “field cancerization” proposed by Slaughter et al.\textsuperscript{34}

This supports the idea that the PI 3-K-initiated signaling pathway is disregulated in the earliest stages of HNSCC development, which might be useful as a molecular biomarker with predictive value for local recurrence or appearance of second primary tumor in the upper aerodigestive tract. Importantly, 17/36 (47%) cases, for which most of the targets of our study were analyzed, harbored at least 1 molecular alteration of PI 3-K-initiated signaling pathway, which makes it one of the most frequently altered in HNSCC, along with p53 deletions/mutations and dysregulation of the G1 to S transition cell cycle checkpoint controlled by cyclin D1 and pRb.

In conclusion, AKT activation, PI 3-K accumulation and PTEN downregulation detected in our HNSCC series are not accounted for by genetic abnormalities (such as AKT2 and PIK3CA gene amplification, allelic losses or homozygous deletions). We propose these could rather reflect early biochemical effects of tobacco components (nicotine) already seen in normal human epithelial cells of the upper aerodigestive tract of HNSCC patients, as has been demonstrated for normal bronchial and lung cancer cells.\textsuperscript{21}

Further studies focused on the roles of nAchRs in AKT activation in HNSCC are warranted.

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