Abstract. Background: Molecular events following nicotinic acetylcholine receptor (nAChR) activation by nicotine are poorly understood. The phosphatidylinositol 3-kinase (PI3-K)/Akt/PTEN pathway has been suggested to play a role in the antiapoptotic responses to nicotine. Materials and Methods: To elucidate the possible role of α3, α5 and α7 nAChR subunit mediated PI3-K/Akt/PTEN pathway activation in squamous cell carcinoma of the head and neck (HNSCC) development, mRNA was isolated from 30 HNSCC tissues of known Akt activation state and were analyzed by reverse transcription polymerase chain reaction (RT-PCR). Results: α3, α5 and α7 nAChR subunits were expressed in 1/30 (3.33%), 15/30 (50%) and 10/30 (33.33%), respectively. These results did not correlate with pAkt levels, previously assessed in our laboratory, or any of the clinicopathological parameters considered. Conclusion: This is the first report on nAChR subunit expression in human HNSCC surgical specimens of known pAkt levels. Our results suggest that nAChRs might exert their function through pathways different from PI3-K/Akt/PTEN and that α3, α5 or α7 nAChR subunit expression might not be useful prognostic markers in HNSCC.

Squamous cell carcinoma of the head and neck (HNSCC) remains a significant cause of morbidity and mortality, affecting approximately 500,000 new cases worldwide each year (1). The complex development of HNSCC is thought to progress through a series of well-defined clinical and histopathological stages, which correlate with the accumulation of multiple genetic events (2). The recognized risk factors for head and neck carcinoma (heavy smoking and high alcohol consumption in developed countries) and the clinical appearance of premalignant lesions are poor predictors of risk of tumor development so molecular markers for cancer-risk assessment are needed (3).

Among the thousands of chemicals present in tobacco smoke, nicotine is one of the main ones responsible for the deleterious consequences of cigarette smoking and has been shown to be involved in the inhibition of apoptosis (4). Moreover, its presence interferes with cell death induced by a variety of DNA-damaging agents, affecting the efficacy of cancer therapy, specifically in head and neck carcinomas (5). Nicotine exerts these effects by displacing the endogenous neurotransmitter acetylcholine (ACh) from the neuronal nicotinic acetylcholine receptors (nAChR) (6). The mammalian neuronal nAChR are a family of proteins each formed by five homologous or identical subunits, arranged symmetrically around a central ion channel. Seven of the subunits harbor the principal components of the ligand binding site (α2, α3, α4, α6, α7, α9, and α10) while four are structural proteins (α5, β2, β3, and β4) that confer unique functional and pharmacological properties on the receptors. Although originally found in neural tissue, nAChRs have recently been reported in non-excitable cell types (6-11), where they might contribute to the development of tobacco-associated morbidity. West et al. (2003) have shown that nicotinic activation of Akt depends upon phosphatidylinositol 3-kinase (PI3-K) and specific nChRs. The signaling pathways from calcium influx to PI3-K/Akt activation have not been well characterized. It has been suggested that the Src family tyrosine kinases may regulate calcium-mediated signaling to the PI3-K/Akt pathway (12). These findings are related to lung cancer, only one of many tobacco-related carcinomas. Nicotinic activation of the PI3-K/Akt pathway might contribute to the biology of other types of tobacco-related tumors. Our laboratory recently identified the PI3-K/Akt/PTEN signaling
pathway as one of the most frequently altered in head and neck squamous cell carcinoma (13), showing that 17/36 (47%) cases for which most of the targets of our study were analyzed harboured at least one molecular alteration of the PI3-K-initiated signaling pathway. Furthermore, it was altered in some normal mucosa paired with our HNSCC series. The role of the nAChR in HNSCC development and its relationship to the PI3-K/Akt pathway nicotine-dependent activation is still unclear. In the present study, the α3, α5 and α7 (previously described to be present in epithelial cell lines) nAChR subunit expression level were investigated in 30 head and neck squamous cell carcinomas, in an effort to determine whether there was any relationship between nAChR expression levels and the PI3-K/Akt pathway activation and whether such expression could be used as molecular markers with any prognostic value.

Materials and Methods

Samples and controls. Surgically derived tissue specimens from 30 patients with HNSCC who consecutively underwent resection of their tumors at the Hospital Universitario Central de Asturias (HUCA) were prospectively obtained for our study, after ethical approval following institutional review board guidelines. None of them had received radio/chemotherapy prior to intervention or were thought to have distant metastasis at the time of diagnosis. Samples were sharply excised, placed in sterile tubes and frozen immediately in liquid nitrogen. All tissue samples were stored at −80°C until RNA extraction and analysis. All cases were confirmed to be neoplastic by the pathologist. The characteristics of the studied patients and the clinicopathological features of their tumors are shown in Table I. All the patients were regular tobacco and alcohol consumers.

The SH-SY5Y neuroblastoma cell line was grown to 80% confluence and total RNA was isolated using Tri-reagent (Sigma, St Louis, MO, USA) following the manufacturer’s protocol and was used as positive control for the α3, α5 and α7 nAChR subunits (14).

RNA isolation. RNA was isolated and purified from 30 samples of fresh frozen tumor tissue using the Micro-to-Midi Total RNA Purification System (Invitrogen Life Technologies, Carlsbad, CA, USA) as specified by the manufacturer, including DNase treatment. The RNA concentration and purity were evaluated by spectrophotometric analysis. The RNA structural integrity was assessed by gel electrophoresis referring to the appearance of rRNA 28S and 18S bands, corresponding to lengths 4.5 and 1.9 kb, respectively, as indicators of absence of RNA degradation.

Detection of nAChR Subunit mRNA by Reverse Transcription Polymerase Chain Reaction (RT-PCR). The total RNA (1 μg) was reverse transcribed using the Thermoscript RT-PCR System (Invitrogen Life Technologies) as specified by the manufacturer. The primers for genes encoding the human nicotinic acetylcholine receptor α3, α5 and α7 subunits were designed with the assistance of the Primer Express software version 2.0 computer program (Applied Biosystems, Foster City, CA, USA). The forward and reverse oligonucleotide sequences and the expected product sizes (in parentheses) were as follows: α3: 5'-AGAGTTCATGCG TGTCCCTG-3' and 5'-AGGCTTTGGTCTTGTCGTCC-3' (101 bp), α5: 5'-GCGCTCGATTCTATTCGCTA-3' and 5'-CGATCA AGAACCTGGGCTATG -3' (101 bp) and α7: 5' ¬CCAATGA CTCGCAACCACTC-3' and 5'-CAGCCAAATGTTGGTGGTT AA-3' (101 bp). Primers forming a pair were placed in different exons to avoid amplification of contaminating genomic DNA. All of them were purchased from Invitrogen Life Technologies. The PCR conditions used were as follows: a 10 min step at 95°C (enzyme activation) followed by 32 cycles of 95°C for 1 min, 60°C (enzyme activation) followed by 32 cycles of 95°C for 1 min, 60°C for 1 min 72°C for 1 min and a final step at 72°C. PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide gel staining.

Statistical analysis. For statistical purposes, clinicopathological features were dichotomized as follows: pT category: 1-3 vs. 4; pN category: 0 (free lymph node) vs. 1-3 (affected lymph node); TNM stage: I-III vs. IV.

The molecular results data distributed among the different clinical groups of tumors were tested for significance employing the χ² test (with Yate’s correction where appropriate) with the help of the statistical software package SPSS 14.0 (SPSS Inc., Chicago, IL, USA). Values for p < 0.05 were considered statistically significant.

Results

α3 subunit mRNA expression was only found in 1/30 cases. Expression of α5 nAChR subunit mRNA was detected in 15/30 (50%) and α7 subunit in 10/30 (33.33%) of the tumors studied.
Interestingly, 7/30 cases (23.3%) co-expressed α5 and α7 subunits. The internal control gene, GAPDH, was expressed at the same relative level in all subjects.

There was no correlation between these results and the pAkt accumulation that had been previously assessed in our laboratory (13) (Table II).

Clinical correlations. No statistical correlation was found between nAChR subunits and any of the clinicopathological features considered (Table III). As could be expected, there was a trend towards patients with tumors arising at the pharynx showing lymph node metastasis more frequently than those with laryngeal tumors (2.983, p = 0.084). In addition, patients with pharyngeal tumors were diagnosed with more advanced TNM stages than those with laryngeal tumors (10.677, p = 0.005).

Discussion

To date, analysis of nAChR subunit expression has been mainly addressed in cell culture or mouse model systems (6, 15-17). This is the first report of the expression of nAChR subunits in a series of human tumor samples (HNSCC) of known Akt activation status. It is well established that nAChRs of different subunit composition exhibit very different pharmacological and functional properties. The fact that the case expressing the α3 subunit also displayed α5 subunit expression is in good agreement with the previous idea that neuronal nicotinic receptors formed by the α3 subunit generally include the α5 and the β2 or β4 subunits (18). The three cases that expressed only the α7 subunit are good candidates to harbour homooligomers of this subunit that have been described to be dramatically different from their heteromeric counterparts. They are more permeable to calcium than other nAChRs which suggests that the activation of the nAChRα7-type receptors, impacts upon free intracellular calcium and calcium-dependent mechanisms in a manner quite distinct from other nAChRs (19). Although α5 subunits are apparently unnecessary for the assembly of functional receptors and cannot yield functional receptors when expressed alone or in combination with β subunits only, they can alter the properties of nAChRs. In addition, functional heteropentamers derived from α7 and α5 have been also reported (19). Our observation that 7/30 of the HNSCC tissues coexpress α5 and α7 subunits raises the possibility that they coassemble, probably with β2, to form functional nAChRs. Seven cases expressed only the α5 subunit. It could participate in receptors containing the α4 and β2 subunits, as has been proposed to explain the central role of α5 in mediating nicotine-induced seizures arising in the hippocampus of α5 −/− mice after short-term exposure to nicotine (20). No significant correlation was found between the expression of any of the studied subunits and any of the clinicopathological parameters considered. Thus, α3, α5 and α7 might not be useful pronostic markers for HNSCC development. Smoking is related to changes in oncogenes or tumor suppressor genes, however, activation of signal transduction pathways that promote cellular survival might also contribute to tobacco-related tumor development. The PI3-K/Akt pathway contributes to tumorigenesis and resistance to therapy and has recently been identified as one of the more frequently altered pathways in HNSCC in our laboratory (13). Many reports have pointed to this pathway as being the mediator of nicotine antiapoptotic effects upon nAChR activation (11, 21). However, the mechanisms through which stimulation of the nAChR results in PI3-K/Akt activation remain obscure. In neuronal systems it appears that nicotine stimulation of the α7 nAChR transduces signals in a cascade to Akt via Janus kinase 2
Other studies have provided evidence for a novel signaling route coupling the stimulation of the α7 nAChR to the activation of extracellular-signal-regulated-kinase (ERK) (23). One study has demonstrated that in human airway epithelial cells Akt is activated upon short-term exposure to nicotine, which antagonizes apoptosis. This effect has been suggested to contribute to tobacco-related carcinogenesis. In addition, Akt activation was found in ten human lung cancer specimens from smokers, but the expression of the nAChR subunits in those samples was not assessed (11). Thus, linking pAkt accumulation to nAChR activation in human lung cancer samples is indirect, as it is based on observations obtained from cultured cells or mouse models. The consequences of nAChR activation are likely to be both cell-type and context specific (24). In our HNSCC series no evidence for any relationship between α3, α5 or α7 expression and Akt pathway activation (previously assessed in our laboratory (13)) was found. The apparent discrepancy between our results and those reported by others could be a consequence of the different effects of transient vs. sustained exposure to nicotine, the latter being the case of every HNSCC patient in our study. In fact, the molecular mechanisms of long-term nicotine exposure in the development of cancer are not clear. The data by Chu et al. (25) have revealed that nicotine potentiates the lung carcinogenesis process, mainly by up-regulating Ras activity. However, they showed that PI3-K, was transiently activated and this effect was only detected after 1 h of nicotine exposure, diminishing after longer exposure periods. Thus, our results suggest that biochemical pathways other than the PI3-K/Akt pathway could be responsible for the adverse effects of nicotine on HNSCC development. Alternatively, differences in technical approaches (western blot vs. immunohistochemical detection of pAkt) could account at least in part for the differing results.

In conclusion, our findings suggest that the adverse long-term effects of tobacco components acting through nAChR activation on the development of HNSCC do not seem to be mediated by the PI3-K/Akt biochemical pathway and that the expression patterns of α3, α5 and α7 might not have prognostic value.

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