

Influence of Tobacco Smoke Exposure on the Protein Expression of $\alpha 7$ and $\alpha 4$ Nicotinic Acetylcholine Receptors in Squamous Cell Carcinoma Tumors of the Upper Aerodigestive Tract (Out of the Larynx)

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ABSTRACT:

PURPOSE: To assess protein expression of $\alpha 7$ and $\alpha 4$ nicotinic acetylcholine receptors (nAChR) subtypes in squamous cell carcinoma of the upper aerodigestive tract (out of the larynx) according to tobacco smoke exposure, considering the general characteristics of the patients.

METHODS: The $\alpha 7$ and $\alpha 4$ nAChR subtypes were assessed by immunohistochemistry in tumor samples from 33 patients with novel diagnosis of squamous cell carcinoma of the upper aerodigestive tract (out of the larynx).

RESULTS: Current smokers were middle-age men with alcohol consumption, whereas elderly women with no alcohol consumption prevailed among nonsmokers. Expression of $\alpha 4$ nAChR was high in all groups, with an influence of alcohol use, although expression of $\alpha 7$ nAChR was low in current smokers with alcohol use. Expression of $\alpha 4$ with no expression of $\alpha 7$ nAChR was associated with advanced disease.

CONCLUSIONS: Squamous cell carcinoma tumors of the upper aerodigestive tract (out of the larynx) may show desensitization of $\alpha 4$ nAChR. Advanced disease at diagnosis might be associated with desensitization of $\alpha 4$ with decrease in $\alpha 7$ nAChR.

KEYWORDS: Receptors, nicotinic, carcinoma, squamous cell, tobacco

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Introduction

Head and neck squamous cell carcinoma is the sixth most common cancer worldwide¹; the main accepted risk factors are tobacco usage and alcohol consumption.² Tobacco is associated with both the development and the progression of cancer.³ Tumor induction may be mediated by tobacco-specific nitrosamines as well as other carcinogens, whereas signaling through the nicotinic acetylcholine receptors (nAChRs) contributes to progression.^{4,5}

Nicotinic acetylcholine receptors are ligand-gated ion channels, activated by acetylcholine, choline, or nicotine that are assembled as complexes.⁶ The $\alpha 4\beta 2$ nAChR and the $\alpha 7$ nAChR are the evolutionarily oldest subtypes.⁷ Although nicotine binds with higher affinity to $\alpha 4\beta 2$ -nAChRs than to $\alpha 7$ nAChRs, after chronic exposure, the higher binding results in long-term inactivation of $\alpha 4\beta 2$ nAChR,⁸ whereas the sensitivity of $\alpha 7$

nAChR may remain unchanged.⁹ Exposure to nicotine or nicotine-derived carcinogenic nitrosamines upregulates cancer-stimulatory nAChR (eg, $\alpha 7$ and $\alpha 9$) and desensitizes cancer-inhibitory nAChR (eg, $\alpha 4\beta 2$).¹⁰ Short-term administration of nicotine can promote angiogenesis and neurogenesis,⁹ whereas chronic exposure may impair cholinergic angiogenesis.¹¹ $\alpha 7$ nAChR is a key regulator of the plasticity of the human airway epithelium by controlling basal cell proliferation and differentiation,¹² and it may be the main nAChR subtype mediating the effects of tobacco on epithelial cells.¹³ In healthy mammals, the stimulatory effects of $\alpha 7$ nAChR are balanced by $\alpha 4\beta 2$ nAChR regulatory effects on γ -amino butyric acid.¹⁴

Clinical studies on the effect of tobacco smoke on nAChR protein expression in squamous cell carcinoma of the aerodigestive tract (out of the larynx) are scarce. In laryngeal and



hypopharyngeal tumors, overexpression of the $\alpha 1$ subunit and downregulation of the $\alpha 3$ and $\alpha 7$ subunits have been described.¹⁵

The aim of this study was to assess the protein expression of $\alpha 7$ and $\alpha 4$ subtypes of nAChR in tumor samples of squamous cell carcinoma of the upper aerodigestive track (out of the larynx) according to tobacco smoke exposure, considering the general characteristics of the patients.

Materials and Methods

The study was approved by the Hospital Committee of Research and Ethics, and written consent was obtained from all the participants. The procedures were performed in accordance with the ethical standards of the World Medical Association Declaration of Helsinki.

In a specialized Medical Center, 33 consecutive patients accepted to participate. They were referred for treatment because of novel diagnosis of squamous cell carcinoma of the upper aerodigestive track (out of the larynx). They had no evidence of systemic infection or anti-inflammatory treatment, none of them had received steroids, radiotherapy, immunotherapy, or chemotherapy. They had no history or evidence of additional cancer, and the diagnosis was confirmed after surgery or biopsy. Women were postmenopausal, but one who was a current smoker and reported alcohol consumption, none of them was receiving sex hormones.

According to exposure to tobacco smoke, which was determined by a validated questionnaire,¹⁶ patients were classified into 3 groups (Table 1).

Group 1: 10 patients (54 ± 2 years old, mean \pm SD) with active exposure during the past 10 to 40 years (20 ± 10 years) (current smokers). They were advised to avoid the exposure during 1 week previous to surgery.

Group 2: 13 patients (73 ± 0 years old) who were exposed during 10 to 40 years (27 ± 7 years) but quitted smoking 2 to 30 years (11 ± 8.9 years) before participating in the study (former smokers).

Group 3: 10 patients (67 ± 10 years old) who denied either active or passive exposure (never smoked).

Tumor samples were obtained before treatment, in the operating room, under general anesthesia with propofol and sevoflurane. Pathology assessment was performed according to the World Health Organization Classification of Tumors.¹⁷ The fragments of tumors were fixed in 10% buffered formalin, paraffin embedded, and stained with hematoxylin-eosin.

Heat-induced epitope retrieval (Nordic Ware, Microwave Tender Cooker cat. NW001-PC; BioGenex, Minneapolis, MN, USA) was performed before assessment of $\alpha 4$ and $\alpha 7$ receptors by immunohistochemistry (Cover plate cat. 72110017 and Rack cat. 73310017; Thermo Scientific, Fremont, CA,

USA); using rabbit polyclonal antibodies (Anti-Nicotinic Acetylcholine Receptor $\alpha 4$ antibody ab41172, 1:25 and $\alpha 7$ antibody ab10096, 1:25; Abcam, Cambridge, UK), and biotin-free polymer detection (MACH 1 Universal HRP-Polymer Detection, cat. M1U539G; Biocare Medical, Concord, CA, USA). To determine the counts of immunoreactive cells per squared millimeter, all samples were analyzed by 2 independent pathologists on the slides of 10 calibrated fields (DM750, $\times 40$; Leica), which were randomly selected.

Statistical analysis was performed according to data distribution and using analysis of variance (with post hoc least significant difference test), χ^2 test, and analysis of covariance (CSS, StatSoft, Tulsa, OK, USA); $P \leq .05$ was considered significant.

Results

The general characteristics of the patients are described in Table 1. Current smokers were younger than former smokers or never smoked ($P < .05$); and the proportion of women was the highest among never smoked ($P < .05$; Table 1). Most of the current smokers and former smokers were alcohol consumers, and those who did not consume alcohol were all women. Although the site of the tumor and the differentiation grade were similar in the 3 groups, among current smokers, there was no patient in stage I at diagnosis, whereas there were the only 2 patients in stage IV at diagnosis (Figure 1A).

Overall, 81.8% of the tumors expressed the $\alpha 4$ subtype, either combined or alone, whereas 48.4% of the tumors expressed the $\alpha 7$ subtype, either combined or alone ($P < .05$). The correlation between the expression of the 2 subtypes was moderated (adjusted $r^2 = .21$, $P < .02$). Counts of the $\alpha 4$ subtype protein were similar in the 3 groups, whereas counts of the $\alpha 7$ subtype were lower among current smokers (Table 1).

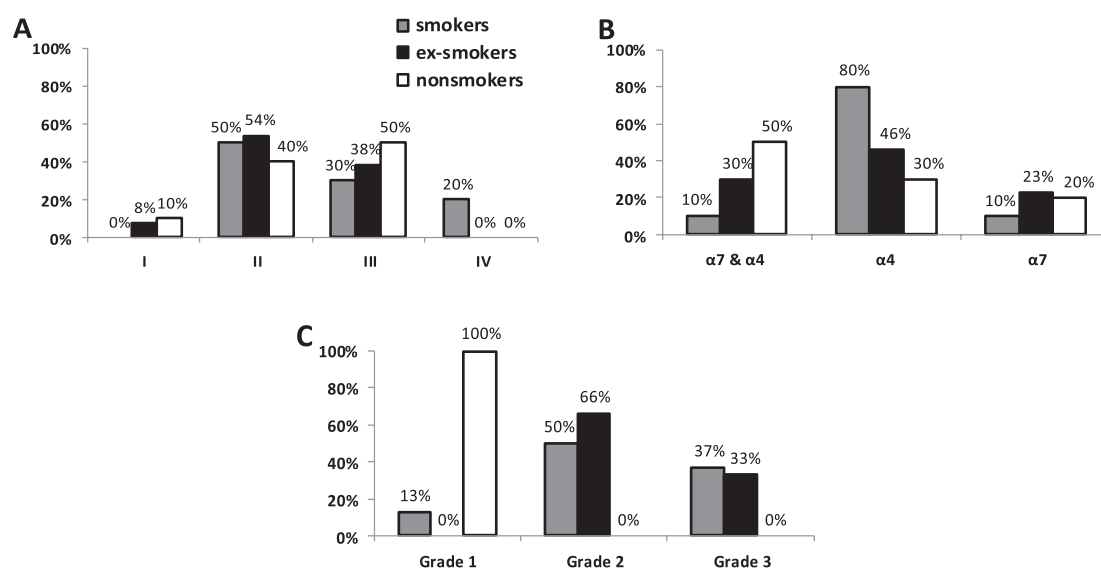
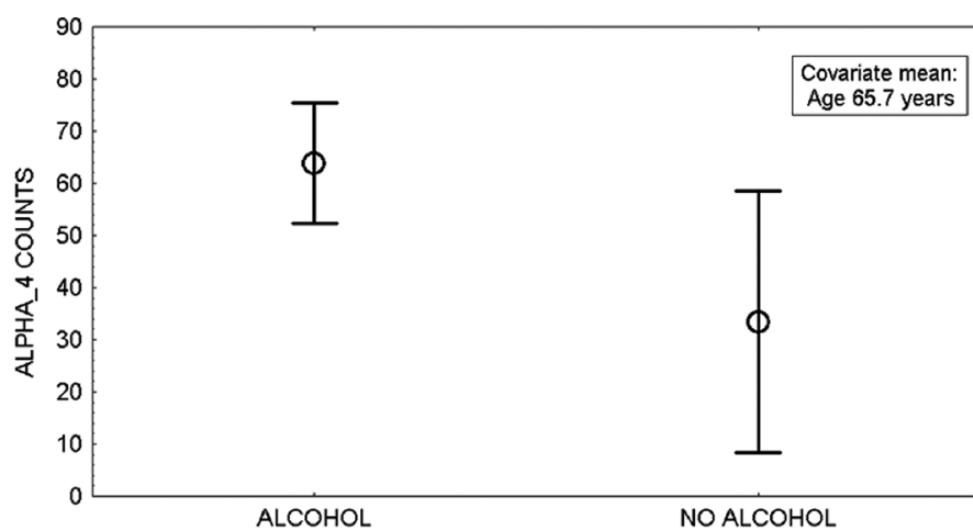
According to tobacco smoke exposure, $\alpha 7$ subtype expression was similar in the 3 groups, whereas expression of both $\alpha 4$ and $\alpha 7$ nAChR was more frequent in the group of those who never smoked (50%) than in the group of current smokers (10%; $P < .05$), with an intermediate frequency in former smokers (30%); and expression of just the $\alpha 4$ subtype was higher in current smokers (80%), compared with those who never smoked (30%; $P < .05$). Higher expression of the $\alpha 4$ subtype was also related to alcohol consumption ($P < .05$; Figure 2). In addition, tumors expressing only the $\alpha 4$ subtype were less differentiated in patients with a history of tobacco smoke exposure than among those who never smoked, in whom they were all grade 1 (3 out of 3; Figure 1C).

Discussion

The results suggest that in squamous cell carcinoma tumors of the upper aerodigestive tract (out of the larynx), $\alpha 4$ nAChR may be highly expressed, in smokers and no smokers, with influence of alcohol consumption, whereas decreased expression of $\alpha 7$ nAChR may be related to the combined effect of current exposure to tobacco smoke and alcohol use.

Table 1. General characteristics of the patients, according to tobacco smoke exposure.

CHARACTERISTIC	SMOKERS (N = 10)	EX-SMOKERS (N = 13)	NONSMOKERS (N = 10)
Age (mean \pm SD in years)	54 \pm 12	73 \pm 10	67 \pm 10
Gender (male/female ratio)	7/3	11/2	4/6
Alcohol consumption (yes/no ratio)	8/2	12/1	5/5
Tumor site (upper airways/oral cavity ratio)	7/3	11/2	7/3
Grade of differentiation			
Grade 1	1	1	4
Grade 2	6	10	5
Grade 3	3	2	1
Counts of α 4 nAChR (mean \pm SD)	58 \pm 24	56 \pm 33	56 \pm 31
Counts of α 7 nAChR (mean \pm SD)	15 \pm 31	29 \pm 29	44 \pm 32

**Figure 1.** According to the group of tobacco smoke exposure. (A) Cancer stage at the time of diagnosis, (B) frequency of protein expression of α 7 and α 4 nAChR, and (C) grade of differentiation of tumors expressing only the α 4 nAChR protein.**Figure 2.** Mean and standard error of the mean of the α 4 nAChR protein counts, computed at mean age, according to consumption or no consumption of alcohol, overall patients.

Experimental evidence support that ethanol by itself may affect expression of nAChRs, depending on the receptor subunit composition,¹⁸ whereas it also affects nicotine-induced signaling processes.^{19,20} Acute exposure to ethanol may blunt the upregulation of $\alpha 4\beta 2$ nAChR¹⁹ and may inhibit $\alpha 7$ subtype.^{18,21,22} In neurons from rats, ethanol also can inhibit $\alpha 7$ nAChRs in concentrations just above the legal limits for intoxication in humans.²² In addition, in this study, expression of $\alpha 4$ nAChR with no expression of $\alpha 7$ nAChR among patients with tobacco smoke exposure and alcohol consumption was associated with more advanced disease. This observation is consistent with the evidence that inactivating $\alpha 7$ nAChR function *in vitro* increases cell proliferation, leading to epithelial alterations such as basal cell hyperplasia and squamous metaplasia,¹³ as well as with the evidence of association between tobacco and alcohol consumption with high frequency of p53 mutations.²³ Although we cannot discard the influence of human papillomavirus infection in the progression of oropharyngeal squamous cell carcinoma,²⁴ its influence on the results of this study may be ameliorated by the low frequency of oral cancer in the sample.

In this study, no influence of advancing age was observed as the protein expression of $\alpha 4$ nAChR was similar in the 3 groups, whereas the lowest frequency of protein expression of $\alpha 7$ nAChR was observed in current smokers, who were the youngest. Most of the current smokers were middle-aged men, who drink, whereas in the never smoking group, elderly women prevailed. The older age observed in never smoked is consistent with previous findings of a lower degree of alcohol and tobacco exposure in elderly patients with head and neck squamous cell carcinoma.²⁵

The finding that smoking female patients with no alcohol consumption could have similar expression of nAChRs than smokers with alcohol intake suggests a possible role of sexual hormones on the expression of nAChR. Progesterone may play a role in tobacco smoking behaviors and it may be an allosteric modulator of nAChRs.²⁶ In immortalized human small airway epithelial cells, chronic exposure to estrogen upregulates and sensitizes $\alpha 7$ nAChR and desensitizes $\alpha 4\beta 2$ nAChR.²⁷ In postmenopause, smokers may show elevated levels of sex hormones compared with nonsmokers,²⁸ whereas during the follicular phase of the menstrual cycle, women who smoke may show increase in progesterone and estradiol.²⁹

Influence from anesthesia on nAChR expression cannot be ignored. Some anesthetic agents may block nAChR *in vitro*. Several studies have shown that halothane, isoflurane, and sevoflurane inhibit the activity of $\alpha 4\beta 2$ nAChR,³⁰ which could have an influence on desensitization and overexpression of the $\alpha 4$ subtype. However, in this study, expression of the $\alpha 7$ subtype was different among the groups, even when anesthetic procedures were similar for all participants.

In conclusion, in squamous cell carcinoma tumors of the upper aerodigestive tract (out of the larynx), $\alpha 4$ nAChR may

be highly expressed with an influence of alcohol use, in both smokers and no smokers, whereas $\alpha 7$ nAChR expression may be decreased in current smokers with alcohol use. Advanced disease at diagnosis might be associated with desensitization of $\alpha 4$ nAChR and no expression of $\alpha 7$ nAChR. Although the sample size of the study allowed comparisons among current and former smokers and those who never smoked, larger studies are needed to elucidate differences among tumor subtypes.

Author Contributions

BBMV: Study design and protocol, evaluation of the patients, data base, processing and analysis of samples, interpretation of results, writing and reviewing of the manuscript.

JCBM: Study design and protocol, evaluation of the patients, data collection, reviewing of the manuscript.

ECV: Study protocol, evaluation of the patients, data collection, reviewing of the manuscript.

FJGV, MSU, CRTM: Study protocol, processing and analysis of samples, interpretation of results, reviewing of the manuscript.

KJR: Study design and protocol, data base, data analysis, interpretation of results, writing and reviewing of the manuscript.

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