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Unbalanced acetylcholinesterase activity in larynx squamous cell carcinoma

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ABSTRACT

Previous reports have demonstrated that a non-neuronal cholinergic system is expressed aberrantly in airways. A proliferative effect is exerted directly by cholinergic agonists through the activation of nicotinic and muscarinic receptors. In cancer, particularly those related with smoking, the mechanism through which tumour cells respond to aberrantly activated cholinergic signalling is a key question. Fifty paired pieces of larynx squamous cell carcinoma and adjacent non-cancerous tissue were compared in terms of their acetylcholinesterase activity (AChE). The AChE activity in non-cancerous tissues (0.248 ± 0.030 milliunits per milligram of wet tissue; mU/mg) demonstrates that upper respiratory tissues express sufficient AChE activity for controlling the level of acetylcholine (ACh). In larynx carcinomas, the AChE activity decreased to 0.157 ± 0.024 mU/mg ($p = 0.009$). Larynx cancer patients exhibiting low ACh-degrading enzymatic activity had a significantly shorter overall survival ($p = 0.031$). Differences in the mRNA levels of alternatively spliced AChE isoforms and molecular compositions were noted between glottic and supraglottic cancers. Our results suggest that the low AChE activity observed in larynx squamous cell carcinoma may be useful for predicting the outcome of patients.

1. Introduction

Head and neck cancer arise in the mucosal layer of the upper aerodigestive tract (oral cavity, oropharynx, hypopharynx, and larynx). Nearly 90% of head and neck carcinomas are squamous cell carcinomas. Head and neck neoplasia is the sixth most frequent cancer, with more than 600,000 new cases reported worldwide each year [1,2]. Larynx cancer is the second most common type of cancer among all head and neck cancers. At the early stage, patients with larynx carcinoma can be cured with multimodal therapy (surgery, radiation, and/or chemotherapy). Unfortunately, no fully satisfactory treatment has yet been developed, and therefore, the mortality rate of larynx carcinoma patients remains high [3]. Reliable biomarkers for distinguishing patients with poor prognosis or risk of early recurrence and for using personalized therapies are still awaited given the uncertainty of the clinical evolution of larynx carcinoma using the current staging criteria.

An increasing body of evidence notes that several cell types are capable of expressing the range of proteins that form a non-neuronal cholinergic system (NNCS), i.e., the acetylcholine (ACh)-synthesizing enzyme choline acetyltransferase (ChAT), nicotinic (nAChR) and muscarinic (mAChR) receptors, and the ACh-hydrolysing enzymes acetyl- (AChE) and butyrylcholinesterase (BChE) [4–7]. A specific cholinergic phenotype depending on the cell type could participate in processes that define a correct tissue physiology. It is worth noting that the results demonstrate that the human respiratory tract epithelium possesses a NNCS engaged in controlling the level of ACh. It appears that this epithelial cholinergic system operates actively to regulate auto/paracrine actions and thereby reliably controls basic cell functions [8]. The proliferative effects arising from cholinergic overactivation have gained basic and translational significance. Of importance is the susceptibility to lung cancer that confers AChR disorders [9] as well as the nicotine-guided shift in the expression of ACh-related proteins to proliferating/migrating cell phenotypes [10]. It is also worth mentioning that promising therapies based in the blockade or attenuation of cholinergic signalling are under investigation [11–13].

The classical function of AChE is to terminate cholinergic transmission through a very efficient hydrolysis of ACh. There are three main alternative splicing forms of AChE: synaptic or tailed AChE (AChE–T),

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erythrocyte or hydrophobic AChE (AChE–H) and read-through AChE (AChE–R). In addition to the hydrolysis of neurotransmitters, AChE has several non-classical roles related with important cell processes such as proliferation, differentiation, apoptosis and cell–cell recognition [14].

The studies on the active involvement of cholinesterases in cell proliferation and differentiation [15] indicate that it is possible that AChE and, to a lower extent, BChE collaborate to tumour development and dissemination. The following examples support this idea: the frequent aberrations in the AChE gene and the structural changes in AChE proteins observed in tumours of diverse origin [8,16–20], the expression of AChE upon apoptosis induction with different stimuli [21–23], and the profitable use of AChE as a prognostic predictor for liver carcinoma and its profitable effects through the suppression of cell growth and the enhancement of chemosensitization [24].

Studies in our laboratory have found that cancer affects the level of AChE activity and/or the content of alternatively spliced mRNAs in the human breast, lymph node, intestine, lung, kidney and prostate. Due to the lack of specific and sensitive biomarkers and tools for early diagnosis, cancer in airways is diagnosed at advanced stages [8,19,25,26]. The aim of this research study was to explore possible changes in the expression of AChE in laryngeal tumours and to test the usefulness of these changes as reliable diagnostic or prognostic markers.

2. Material and methods

2.1. Patients and samples

A total of 50 human malignant primary larynx squamous cell carcinomas (LSCCs) and their adjacent noncancerous tissues (ANCT) collected through surgeries at Virgen de la Arrixaca Clinical University Hospital in Murcia (Spain) from 2007 to 2012 were included in the current study. Fresh specimens were divided into sections and stored at −80 °C until use. The TNM classification of LSCC specimens was made according to the UICC:TNM Classification of Malignant Tumors. Study approval and the consent procedure were obtained from the Institutional Ethic Committee of our Hospital. All of the patients gave their consent after being appropriately informed.

2.2. Extraction and assay of acetylcholinesterase

AChE was extracted from surgical ANCT and LSCC pieces through homogenization (5% w/v) with Tris-saline buffer (TSB; 1 M NaCl, 50 mM $MgCl_2$, 3 mM EDTA, 10 mM Tris, pH 7.0) supplemented with 1% Brij 96 and a fresh mixture of proteinase inhibitors (0.1 mg/ml soybean trypsin inhibitor, 1 mg/ml bacitracin, 0.0022 TIU/ml aprotinin, 10 mg/ml pepstatin A and 20 mg/ml leupeptin). After centrifugation at 30,000 rpm and 4 °C for 1 h with a 70Ti Beckman rotor (Palo Alto, CA, USA), the supernatant with AChE was saved. The AChE activity was determined as described previously [8]. AChE activity is given in milliunits per milligram of wet tissue (mU/mg) indicating that 1 nmol of substrate is hydrolysed per minute and per milligram of wet tissue.

2.3. Sedimentation analysis

Possible differences between ANCT and LSCC in the molecular distribution of AChE were tested through sedimentation analysis with sucrose gradients as reported previously [8]. Briefly, the samples and sedimentation markers (bovine liver catalase and intestine alkaline phosphatase) were layered on the top of centrifuge tubes containing 5–20% sucrose gradients in the presence of 0.5% w/v Brij 96 detergent. The gradient tubes were centrifuged at 35,000 rpm and 4 °C for 18 h with a SW41Ti rotor in a Beckman L-80 OPTIMA XP Ultracentrifuge (Fullerton, CA, USA). After centrifugation, fractions of 250 μl were collected from the tube bottom and assayed for AChE activity and enzyme markers.

2.4. mRNA isolation and real-time PCR

Differences between ANCT and LSCC specimens in the expression level of AChE mRNAs were studied by RT-PCR. To achieve this, the mRNA from tissues was extracted using the Chemagic mRNA Direct Kit (Chemagen) and reversed transcribed into cDNA by random priming (GeneAmp RNA PCR Kit, Applied Biosystems). A LightCycler thermocycler (Roche Molecular Biochemicals, Mannheim, Germany) was used for RT-PCR. Pairs of primers were designed for quantitative PCR targeting the 3′-alternative mRNAs of AChE with the following sequences: AChE-T, 5′-AACTTTGCCCGCACAGGGGA-3′ (sense) and 5′-GCCTCGTCGAGCGTGTCGGT-3′ (antisense); AChE-H, 5′-AACTTTGCCCGCACAGGGGA-3′ (sense) and 5′-GGGAGCCTCCGAGGCGGT-3′ (antisense); AChE-R, 5′-CCCCTGGACCCCTCTCGAAAC-3′ (sense) and 5′-ACCTGGCGGGCTCCCACTC-3′ (antisense); and BChE, 5′-TGCAAAATATGGGAATCCAAA-3′ (sense) and 5′-CCACTCCCATTCTGCTTCAT-3′ (antisense). β-actin mRNA was used as a housekeeper gene with the primers 5′-AGAAAATCTGGCACCACACC-3′ (sense) and 5′-GGGGTGTTGAAGGTCTCAAA-3′ (antisense). The reaction conditions were validated separately for each pair of primers, and a single dissociation curve peak was produced in each reaction run. The buffered medium contained 5 μL of variable dilutions of cDNA, 0.3 μM specific primers, and an appropriate volume of PCR master mix to obtain a final volume of 20 μL. The reactions comprised a first step of 10 min at 95 °C followed by 40 cycles of 10 s to 95 °C, 10 s at 60 °C, and 15 s at 72 °C. A final dissociation stage allowed us to study the melting curves. The relative content of cDNAs with respect to β-actin cDNA was determined by the second derivative method with kinetic PCR efficiency correction. The PCR products were separated in 2% agarose gels and visualized with ethidium bromide to ensure that their lengths coincided with the expected size. Negative controls (without reverse transcriptase) for each primer pair were also prepared. The relative mRNA amounts are given as the numbers of copies per million of the β-actin mRNA copies.

2.5. Statistical analysis

The results are given as the means ± SEM from 50 paired ANCT and LSCC samples performed at least in triplicate. The numerical data were analysed for statistical significance using the Wilcoxon signed-rank test. The statistical significance of the differences in the mean values was set to $p < 0.05$. Kaplan–Meier curves were constructed to estimate the overall survival, and the differences between the curves were assessed using a two-tailed log-rank test. A difference with $p < 0.05$ was considered to be statistically significant. The data were analysed using the SPSS software, version 11.5 (SPSS Inc., Chicago, IL, USA).

Table 1
Summary of demographic characteristics of LSCC patients and AChE activity.

Samples		AChE activity (mU/mg tissue)		
	N	ANCT	LSCC	*P*-value
Total	50	0.248 ± 0.030	0.157 ± 0.024	**0.009**
Age				
<60	15	0.299 ± 0.069	0.192 ± 0.045	**0.016**
>60	35	0.214 ± 0.029	0.170 ± 0.031	0.066
Tobacco				
Non-smoker	5	0.272 ± 0.092	0.221 ± 0.056	0.347
Former-smoker	23	0.158 ± 0.025	0.204 ± 0.042	0.750
Active-Smoker	18	0.222 ± 0.059	0.148 ± 0.040	**0.002**
Alcohol				
No	16	0.157 ± 0.025	0.193 ± 0.062	0.305
Yes	15	0.340 ± 0.083	0.129 ± 0.030	**0.047**
Location				
Glottis	29	0.226 ± 0.039	0.167 ± 0.029	**0.020**
Supraglottis	21	0.275 ± 0.032	0.141 ± 0.023	**0.005**

LSCC, larynx squamous cell carninoma. ANCT, adjacent non-cancerous tissue.
Statistically significant differences are in bold.