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ORIGINAL ARTICLE

Retrospective Cohort Study

Nicotinic cholinergic receptors in esophagus: Early alteration during carcinogenesis and prognostic value

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Abstract

AIM: To compare expression of nicotinic cholinergic receptors (*CHRNs*) in healthy and squamous cell carcinoma-affected esophagus and determine the prognostic value.

METHODS: We performed RT-qPCR to measure the expression of *CHRNs* in 44 esophageal samples from healthy individuals and in matched normal surrounding mucosa, and in tumors from 28 patients

diagnosed with esophageal squamous cell carcinoma (ESCC). Next, we performed correlation analysis for the detected expression of these receptors with the habits and clinico-pathological characteristics of all study participants. In order to investigate the possible correlations between the expression of the different CHRN subunits in both healthy esophagus and tissues from ESCC patients, correlation matrices were generated. Subsequently, we evaluated whether the detected alterations in expression of the various *CHRNs* could precede histopathological modifications during the esophageal carcinogenic processes by using receiver operating characteristic curve analysis. Finally, we evaluated the impact of *CHRNA5* and *CHRNA7* expression on overall survival by using multivariate analysis.

RESULTS: *CHRNA3*, *CHRNA5*, *CHRNA7* and *CHRNB4*, but not *CHRNA1*, *CHRNA4*, *CHRNA9* or *CHRNA10*, were found to be expressed in normal (healthy) esophageal mucosa. In ESCC, *CHRNA5* and *CHRNA7* were overexpressed as compared with patient-matched surrounding non-tumor mucosa (ESCC-adjacent mucosa; $P < 0.0001$ and $P = 0.0091$, respectively). Positive correlations were observed between *CHRNA3* and *CHRNB4* expression in all samples analyzed. Additionally, *CHRNB4* was found to be differentially expressed in the healthy esophagus and the normal-appearing ESCC-adjacent mucosa, allowing for distinguishment between these tissues with a sensitivity of 75.86% and a specificity of 78.95% ($P = 0.0002$). Finally, *CHRNA5* expression was identified as an independent prognostic factor in ESCC; patients with high *CHRNA5* expression showed an increased overall survival, in comparison with those with low expression. The corresponding age- and tumor stage-adjusted hazard ratio was 0.2684 (95%CI: 0.075-0.97, $P = 0.0448$).

CONCLUSION: Expression of *CHRNs* is homogeneous along healthy esophagus and deregulated in ESCC, suggesting a pathogenic role for these receptors in ESCC development and progression.

Key words: Nicotinic cholinergic receptors; Esophagus; Esophageal squamous cell carcinoma; Tobacco; Alcohol; Gene expression

Core tip: Esophageal squamous cell carcinoma (ESCC) is the main histological subtype of esophageal cancer, and is associated with alcohol and tobacco consumption. Tobacco components, such as nicotine and nitrosamines, are high-affinity agonists of nicotinic cholinergic receptors (CHRNs), the activation of which triggers cellular signaling pathways important for cancer progression. However, data regarding differential expression and regulation of CHRNs in healthy esophageal mucosa and ESCC are limited. This study shows homogeneous expression of *CHRNs* along healthy esophagus and deregulation in ESCC, *CHRNB4* overexpression preceding the first histopathological alterations during ESCC development, and *CHRNA5* expression as an independent predictor of prognosis.

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INTRODUCTION

Worldwide, esophageal cancer (EC) is the 8th most frequent type of cancer and the 6th most common cause of cancer-related deaths[1], reflecting the high mortality rate associated with the disease that is a direct consequence of late diagnosis and poor treatment response[1-4]. Prognosis of EC patients is directly affected by tumor invasion and since dissemination occurs very early during the natural history of the disease due to the lack of serosa in the esophagus, identifying the mechanisms involved in this process is of major interest[5].

Esophageal squamous cell carcinoma (ESCC) is the main histological type of EC, accounting for about 90% of all EC cases globally[2]. The highest incidence rates of EC occur in developing countries, such as Brazil, where ESCC is also the most common histological subtype[2,6]. Several epidemiological studies have indicated that alcohol consumption and tobacco smoking are the major risk factors for ESCC development[7-9]. Studies from Western countries have shown that the concomitant use of these products multiplies the risk for disease development, with tobacco smoking identified as an important contributor to both tumor initiation and promotion and alcohol characterized as acting as a tumor promoter primarily[7,8,10].

Cigarette smoke contains potent carcinogens, such as polycyclic aromatic hydrocarbons and nitrosamines, which have been demonstrated extensively as associated with induction of different types of tumors[11]. These compounds are capable of inducing DNA adducts and mutations and, therefore, have been suggested to participate in the initiation of tobacco-related cancers[11]. However, tobacco-specific nitrosamines and nicotine itself may contribute to tumorigenesis through other additional mechanisms[12-16]. Activation of the nicotinic cholinergic receptors (CHRNs) by these compounds is known to trigger cellular signaling pathways that play key roles in cancer progression, including cell proliferation, angiogenesis, apoptosis inhibition and cell migration[16]. Therefore, researchers have put forth extensive efforts towards characterizing the role(s) of CHRNs in tobacco-related tumors. Most of the studies

to date have focused on lung cancer[17-19], and the results have shown that lung tumors lack expression of *CHRNA3*, due to promoter hypermethylation, but have overexpression of *CHRNA5* and *CHRNA7*[17,19]. It has also been shown that nicotine can modulate the expression of CHRNs in lung cells[20,21]. Finally, in addition to stimulation of cell proliferation and survival, the nicotine-induced activation of CHRNs was also shown to stimulate epithelial-mesenchymal transition and to promote invasion of lung cancer cell lines as well as of cells derived from breast and pancreatic tumors[21].

Based on these findings, it has been proposed that nicotine is likely to contribute to the progression of tobacco-related cancers through binding to CHRNs and the consequent activation of cellular pathways involved in tumorigenesis. Therefore, we hypothesized that alterations in CHRN expression may take part in and contribute to the pathogenesis of ESCC. Since there are no detailed data regarding CHRN expression in human esophagus, this study was designed to determine the expression profile of these receptors in healthy esophagus and ESCC to explore the role of these receptors in this type of cancer.

MATERIALS AND METHODS

Samples

Forty-four fresh esophageal samples were obtained from healthy donors undergoing endoscopy at the Hospital Universitário Pedro Ernesto (HUPE-UERJ, Rio de Janeiro, Brazil) for use, with informed consent, in this study. None of these volunteer donors showed any observable alterations in the esophageal structure at the time of the biopsy and none had a history of cancer. Samples were collected from each third of the esophagus (upper, middle and lower). Furthermore, esophageal samples were obtained from 28 patients with a confirmed diagnosis of ESCC, but who had not undergone any treatment at the time of biopsy at the Instituto Nacional do Câncer (INCA; Rio de Janeiro, Brazil) for use, with informed consent, in this study; for all patients, tumor and matched histologically normal surrounding mucosa (taken 4 inches from the tumor border) were collected and stored at the National Tumor Bank of the INCA (BNT/INCA).

Clinical and demographic data were obtained from the hospitals' medical records for all study participants. Healthy donors provided additional data by answering a standardized questionnaire. All participants signed an informed consent form prior to study enrollment. The project was approved by the Ethics Committees of all involved institutions.

RNA extraction

Total RNA was extracted from the respective samples by using the TRIzol® reagent (Invitrogen, United States), following the manufacturer's protocol. All RNA samples were quantified by spectrophotometry and purity was verified by calculating the absorbance ratio of 260 nm/280 nm and ensuring the ratio was $\geqslant$ 1.7.

Reverse transcription-quantitative PCR

A total of 500 ng RNA was reverse transcribed using SuperScript II® (Invitrogen) and following the manufacturer's protocol. The Rotor-Gene *Q* system (Qiagen, Germany) and QuantiFast SYBR Green PCR Kit (Qiagen) were used for the qPCR, and each reaction was optimized for the particular pair of primers comprising exon-exon junctions to evaluate the mRNA expression of CHRN subunits (Table 1)[20,22]. *GAPDH* served as the reference gene. Each reaction contained 7.5 μL of QuantiFast SYBR Green Buffer × 2 (Qiagen), specific oligonucleotides at a final concentration of 0.5 μmol/L, 1 μL of cDNA (diluted × 10) and sterile deionized water to complete the final volume of 15 μL. The amplification reaction was performed as follows: 5 min of pre-denaturation at 95 ℃, followed by 40 cycles of denaturation for 5 s at 95 ℃ and an annealing and extension step for 10 s at 60 ℃. For *CHRNA1* and *CHRNA9*, annealing steps of 10 s at 58.1 ℃ and of 5 s at 62 ℃ were added, respectively; finally, an extension step of 10 s at 60 ℃ was performed for both of these subunits. After the reaction, the expression of each CHRN subunit was normalized to the *GAPDH* expression, using the comparative Ct method[23].

Statistical analysis

All statistical analyses were performed using the GraphPad Prism 5 software (GraphPad Software, United States). Differences were considered statistically significant when P was < 0.05. When comparing two groups, the unpaired t test or Mann-Whitney test was used. For comparison of paired samples, the paired t test or Wilcoxon signed rank test was applied. For determining significant differences of mRNA expression (expressed as medians) among the different groups of samples, the one-way ANOVA or Kruskal Wallis test and Tukey's post-test or Dunn's post-test was used.

Correlations between the expression levels of the different genes were determined using Pearson's or Spearman's correlation tests. A receiver operating characteristic (ROC) curve was plotted to determine the value of gene expression as a marker to distinguish healthy esophagus from normal-appearing ESCC-adjacent mucosa.

For the estimate of univariate survival, a Kaplan-Meier survival curve was generated and the statistical significance between the two groups was calculated using the log-rank test. Variables that have been shown to influence ESCC outcome, such as age and tumor stage, were selected for the multivariate analysis. Finally, we applied Cox regression using the stepwise forward method. All survival analyses were performed using the software package "Survival" in the R statistical program[24]. The statistical methods of this study were reviewed by Dr. Mariana Boroni from INCA (Rio de Janeiro, Brazil).

Table 1 Sequences of the oligonucleotides used in reverse transcription-quantitative PCR

Gene	Oligonucleotide sequences (5'-3')	Positive control	Ref.
CHRNA1	Forward: ACCAGGAGTCTAACAATGCG Reverse: ACAAGCATGAAGACTCCGAG	Glioblastoma cell line, U251	Designed by the authors
CHRNA3	Forward: AACCTGTGGCTCAAGCAAATCT Reverse: CATGAACTCTGCCCCACCAT	Lung cancer cell line, A549	[20]
CHRNA4	Forward: ACACAGACTTCTCGGTGAAG Reverse: CAGCAGGCAGACGATGATGA	ESCC cell line, TE1	Designed by the authors
CHRNA5	Forward: AGATGGAACCCTGATGACTATGGT Reverse: AAACGTCCATCTGCATTATCAAAC	Lung cancer cell line, A549	[20]
CHRNA7	Forward: GCTGCTCGTGGCTGAGATC Reverse: TGGCGAAGTACTGGGCTATCA	Lung cancer cell line, A549	[20]
CHRNA9	Forward: GATGGCCTAGACTCCATCAG Reverse: CTGAAGATTCATCATCAGCCTTG	Glioblastoma cell line, U251	Designed by the authors
CHRNA10	Forward: CTACTCCCTGCAGAGTGCCTG Reverse: TCTGGTCTGTGTCTGCCACAG	Glioblastoma cell line, U251	Designed by the authors
CHRNB4	Forward: TCACAGCTCATCTCCATCAAGCT Reverse: CCTGTTTCAGCCAGACATTGGT	Lung cancer cell line, A549	[20]
GAPDH	Forward: CAACAGCCTCAAGATCATCAGCAA Reverse: AGTGATGGCATGGACTGTGGTCAT	-	[22]

ESCC: Esophageal squamous cell carcinoma.

Table 2 Characterization of the healthy individuals and esophageal squamous cell carcinoma patients

Evaluated criteria	Number of individuals (range or %)	
	Healthy individuals, *n* = 44	ESCC patients, *n* = 28
Age (yr)	56.50 (18-85)	59.50 (46-79)
Sex		
Men	13 (29.5)	18 (64.3)
Women	31 (70.5)	10 (35.7)
Smoking		
Never	27 (61.4)	1 (3.6)
Ever	17 (38.6)	27 (96.4)
Alcohol consumption		
Never	20 (45.5)	2 (7.1)
Ever	24 (54.5)	26 (92.9)
Death		
No	NA	8 (28.6)
Yes	NA	20 (71.4)
Tumor localization		
Upper third	NA	9 (32.1)
Middle third	NA	15 (53.6)
Lower third	NA	3 (10.7)
Histological grade, differentiation		
Poor	NA	6 (21.4)
Moderate	NA	21 (75.0)
Well	NA	1 (3.6)
Tumor stage		
1 + 2	NA	7 (25.0)
3 + 4	NA	15 (53.6)
Not determined	NA	6 (21.4)

One patient had the tumor located in the middle/lower third of the esophagus (3.6%). NA: Not applicable.

RESULTS

Clinical and demographic characteristics of the study participants

Table 2 shows that most of the healthy individuals were women (70.5% of the group), never smokers (61.4%) and ever drinkers (54.5%), with a median age of 56.5 years (range, 18-85 years). By comparison, the ESCC patients were mostly male (64.3%), ever smokers (96.4%) and ever drinkers (92.9%), with a median age of 59.5 years (range, 46-79 years). Most of the ESCC patients died as a consequence of the disease (71.4%), with most of the tumors affecting the middle third of the esophagus (53.6%), being moderately differentiated (75.0%), and corresponding to stages 3 and 4 (53.6%).

Expression of CHRN subunits in healthy esophagus samples and correlation with clinical and demographic characteristics

Figure 1 shows that *CHRNA3*, *CHRNA5*, *CHRNA7* and *CHRNB4* expression was detected in all healthy esophagus samples evaluated and that the expression levels did not differ significantly among the esophageal thirds nor according to the smoking and drinking status of the individuals (Kruskal Wallis test and Dunn's post-test). There was no association found between the *CHRN*s expression and age, sex, smoking status or alcohol consumption (Table 3). Expression of *CHRNA1*, *CHRNA4*, *CHRNA9* and *CHRNA10* was undetectable in the healthy esophagus samples.

Expression of CHRN subunits in ESCC samples and correlation with clinical and demographic characteristics

Figure 2 shows that *CHRNA5* and *CHRNA7* expression was higher in ESCC samples than in either the matched adjacent mucosa ($P < 0.0001$ and $P = 0.0091$, respectively; Wilcoxon signed rank test) or the esophageal mucosa samples from healthy individuals ($P = 0.0157$ and $P = 0.0004$, respectively; Mann-Whitney test). In addition, *CHRNB4* expression was higher in both tumor samples and the matched surrounding mucosa than in the esophagus samples from healthy

Table 3 Association of *CHRN* expression with characteristics of healthy individuals

Variable	*CHRNA3*		*CHRNA5*		*CHRNA7*		*CHRNB4*	
	Expression, median (min-max)	*P* value	Expression, median (min-max)	*P* value	Expression, median (min-max)	*P* value	Expression, median (min-max)	*P* value
Esophageal thirds								
Upper	1.563×10^{-5} (2.735×10^{-6} - 7.183×10^{-5})	0.769	8.431×10^{-5} (1.277×10^{-5} - 2.157×10^{-3})	0.945	1.023×10^{-5} (1.033×10^{-6} - 5.616×10^{-5})	0.789	9.027×10^{-6} (2.534×10^{-6} - 2.828×10^{-5})	0.153
Middle	1.783×10^{-5} (1.512×10^{-6} - 1.196×10^{-4})		8.476×10^{-5} (1.286×10^{-5} - 2.388×10^{-3})		1.346×10^{-5} (2.588×10^{-6} - 6.319×10^{-5})		1.077×10^{-5} (3.913×10^{-6} - 3.259×10^{-5})	
Lower	1.721×10^{-5} (2.914×10^{-6} - 6.796×10^{-5})		8.906×10^{-5} (1.442×10^{-5} - 1.990×10^{-3})		1.263×10^{-5} (1.678×10^{-6} - 5.636×10^{-5})		1.166×10^{-5} (3.621×10^{-6} - 4.453×10^{-5})	
Age, yr	58		56.5		57		57	
> median	2.226×10^{-5} (1.512×10^{-6} - 1.019×10^{-4})	0.422	4.982×10^{-5} (1.286×10^{-5} - 2.388×10^{-3})	0.097	1.189×10^{-5} (2.588×10^{-6} - 4.391×10^{-5})	0.272	1.060×10^{-5} (3.913×10^{-6} - 3.192×10^{-5})	0.778
⩽ median	1.274×10^{-5} (3.977×10^{-6} - 1.196×10^{-4})		9.054×10^{-5} (2.657×10^{-5} - 4.635×10^{-4})		1.449×10^{-5} (2.931×10^{-6} - 6.319×10^{-5})		1.094×10^{-5} (5.010×10^{-6} - 3.259×10^{-5})	
Sex								
Male	1.344×10^{-5} (3.977×10^{-6} - 5.947×10^{-5})	0.253	1.104×10^{-4} (2.657×10^{-5} - 3.351×10^{-4})	0.243	1.316×10^{-5} (2.931×10^{-6} - 4.060×10^{-5})	0.807	1.772×10^{-5} (5.559×10^{-6} - 2.917×10^{-5})	0.372
Female	1.812×10^{-5} (1.512×10^{-6} - 1.196×10^{-4})		7.842×10^{-5} (1.286×10^{-5} - 2.388×10^{-3})		1.375×10^{-5} (2.588×10^{-6} - 6.319×10^{-5})		1.034×10^{-5} (3.913×10^{-6} - 3.259×10^{-5})	
Smoking								
Never	1.665×10^{-5} (1.512×10^{-6} - 1.019×10^{-4})	0.400	8.531×10^{-5} (2.051×10^{-5} - 2.388×10^{-3})	0.961	1.210×10^{-5} (2.588×10^{-6} - 6.319×10^{-5})	0.582	1.077×10^{-5} (3.913×10^{-6} - 3.192×10^{-5})	0.599
Ever	1.948×10^{-5} (5.157×10^{-6} - 1.196×10^{-4})		8.405×10^{-5} (1.286×10^{-5} - 3.351×10^{-4})		1.375×10^{-5} (2.931×10^{-6} - 4.256×10^{-5})		1.078×10^{-5} (5.010×10^{-6} - 3.259×10^{-5})	
Alcohol consumption								
Never	2.386×10^{-5} (1.512×10^{-6} - 1.196×10^{-4})	0.163	5.955×10^{-5} (2.051×10^{-5} - 3.525×10^{-4})	0.131	1.046×10^{-5} (2.588×10^{-6} - 4.256×10^{-5})	0.412	1.094×10^{-5} (3.913×10^{-6} - 3.259×10^{-5})	0.521
Ever	1.274×10^{-5} (3.977×10^{-6} - 8.783×10^{-5})		1.018×10^{-4} (1.286×10^{-5} - 2.388×10^{-3})		1.375×10^{-5} (2.931×10^{-6} - 6.319×10^{-5})		1.007×10^{-5} (5.010×10^{-6} - 2.917×10^{-5})	

Statistical analyses were performed using the Mann-Whitney or Kruskal Wallis test and Dunn's post-test as appropriate; no significant differences were observed.

individuals ($P = 0.0180$ and $P = 0.0005$, respectively; Mann-Whitney test). The expression of *CHRNA3* was similar in tumor, matched surrounding tissue (Wilcoxon signed rank test) and healthy esophageal mucosa (Mann-Whitney test).

Correlation matrices showed positive associations between the expression of *CHRNA3* and *CHRNB4* in the healthy esophagus samples ($r = 0.47$, $P = 0.007$, Spearman's correlation test), in the normal surrounding mucosa samples from ESCC patients ($r = 0.607$, $P = 0.006$, Spearman's correlation test) and in the tumor tissues from the ESCC patients ($r = 0.544$, $P = 0.016$, Spearman's correlation test) (Table 4). Additionally, a positive correlation was shown to exist between the expression of *CHRNB4* and *CHRNA5* in the normal surrounding mucosa from ESCC patients ($r = 0.556$, $P = 0.013$; Spearman's correlation test). Finally, the expression of *CHRNA3* and *CHRNA7* ($r = 0.511$, $P = 0.026$, Spearman's correlation test) and of *CHRNB4* and *CHRNA7* ($r = 0.561$, $P = 0.012$, Spearman's correlation test) were shown to be positively correlated in tumors.

Evaluation of the potential association between the fold-change (ratio of mRNA expression between tumor and matched surrounding tissue) of the expression of the different subunits and the clinicopathological data indicated no statistically significant associations (Table 5).

Distinguishment of healthy esophagus from ESCC-adjacent non-tumor mucosa by CHRNB4 expression

We next analyzed whether altered expression of *CHRNs* could precede histopathological modifications during esophageal carcinogenesis. Interestingly, the expression of *CHRNB4* was able to distinguish the normal-appearing surrounding tissue of ESCC patients from the esophageal mucosa of healthy individuals.

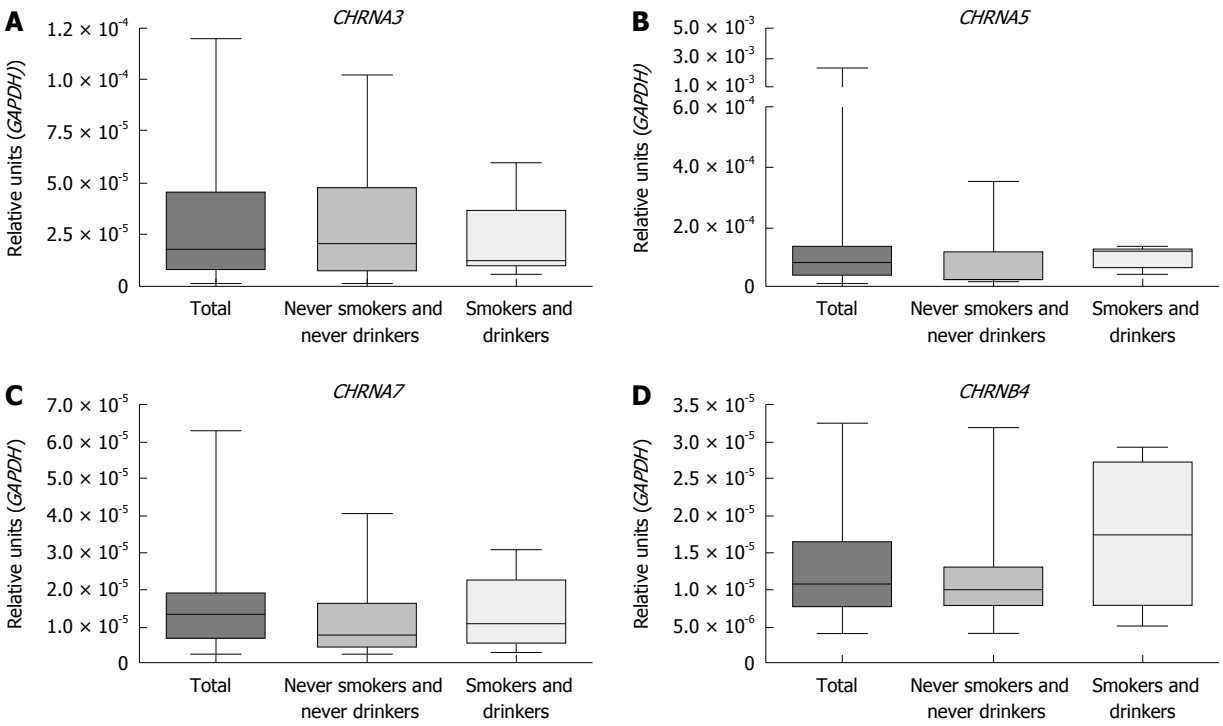


Figure 1 Comparison of *CHRNs*' expression in esophageal samples from healthy individuals. A: Evaluation by RT-qPCR of the mRNA expression of *CHRNA3*; B: *CHRNA5*; C: *CHRNA7* and D: *CHRNB4* in esophageal epithelium from healthy individuals (total individuals, never smokers and never drinkers, current smokers and current drinkers).

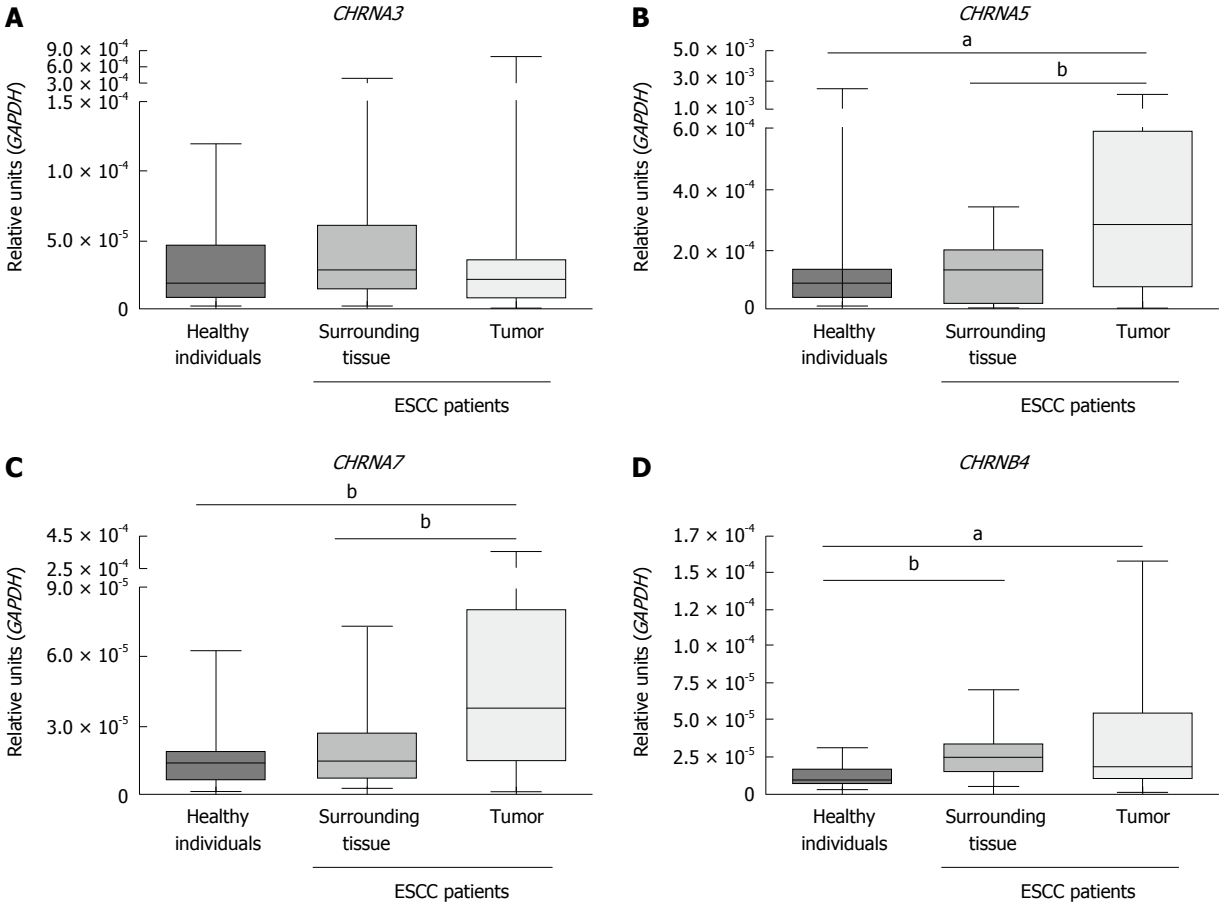


Figure 2 Comparison of *CHRNs*' expression in esophageal samples from healthy individuals and esophageal squamous cell carcinoma patients. A-D: Evaluation by RT-qPCR of the mRNA expression of *CHRNA3* (A), *CHRNA5* (B), *CHRNA7* (C) and *CHRNB4* (D) in esophageal epithelium from healthy individuals and ESCC patients (normal surrounding mucosa and tumor tissue). $^{a}P < 0.05$; $^{b}P < 0.01$.

Table 4 Correlation matrices between the mRNA expression of *CHRN* subunits in healthy esophagus and esophageal squamous cell carcinoma tissues

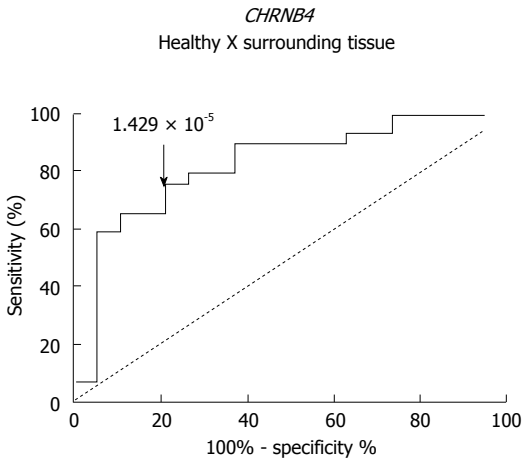
		CHRNA3	*CHRNA5*	*CHRNA7*	*CHRNB4*
Healthy esophagus	*CHRNA3*		$P = 0.820$	$P = 0.203$	$P = 0.007$[a]
	CHRNA5	$r = -0.040$		$P = 0.727$	$P = 0.672$
	CHRNA7	$r = 0.220$	$r = 0.060$		$P = 0.193$
	CHRNB4	$r = 0.470$	$r = -0.079$	$r = 0.240$	
Normal surrounding mucosa	*CHRNA3*		$P = 0.486$	$P = 0.753$	$P = 0.006$[a]
	CHRNA5	$r = 0.170$		$P = 0.847$	$P = 0.013$[a]
	CHRNA7	$r = -0.077$	$r = -0.038$		$P = 0.185$
	CHRNB4	$r = 0.607$	$r = 0.556$	$r = -0.318$	
Tumor Tissue	*CHRNA3*		$P = 0.450$	$P = 0.026$[a]	$P = 0.016$[a]
	CHRNA5	$r = 0.184$		$P = 0.130$	$P = 0.071$
	CHRNA7	$r = 0.511$	$r = 0.293$		$P = 0.012$[a]
	CHRNB4	$r = 0.544$	$r = 0.423$	$r = 0.561$	

Matrices show the *P* values and correlation coefficients between the mRNA expression of the different CHRN subunits in the healthy esophagus, in the surrounding mucosa of esophageal squamous cell carcinoma (ESCC) patients and in ESCC. [a]$P < 0.05$.

Table 5 Association of *CHRN* expression fold-change and clinicopathological data of esophageal squamous cell carcinoma patients

Clinicopathological data	*CHRNA3*		*CHRNA5*		*CHRNA7*		*CHRNB4*	
	FC, median (min-max)	*P* value	FC, median (min-max)	*P* value	FC, median (min-max)	*P* value	FC, median (min-max)	*P* value
Age, yr	60 (46-79)		59.5 (46-79)		59.5 (46-79)		60 (46-79)	
> median	1.1 (0.03136-14.69)	0.2775	2.166 (0.3918-15.07)	0.1612	2.867 (0.2630-20.53)	0.9451	1.984 (0.1586-7.119)	0.1128
≤ median	0.5844 (0.006324-10.62)		3.193 (0.8615-11.66)		1.267 (0.4813-23.10)		0.7518 (0.1414-2.051)	
Sex								
Men	0.9777 (0.006324-14.69)	0.1053	3.032 (0.3918-15.07)	0.1719	2.867 (0.4813-23.10)	0.7192	1.685 (0.2261-7.119)	0.1053
Women	0.1153 (0.03349-1.072)		2.102 (0.5510-3.593)		1.657 (0.2630-20.53)		0.3153 (0.1414-2.809)	
Smoking								
Never	NA	NA	NA	NA	NA	NA	NA	NA
Ever	0.6941 (0.006324-14.69)		3.01 (0.3918 - 15.07)		1.86 (0.2630-23.10)		1.229 (0.1414-7.119)	
Alcohol consumption								
Never	NA	NA	NA	NA	NA	NA	NA	NA
Ever	0.5844 (0.006324-14.69)		2.971 (0.3918-15.07)		1.657 (0.2630-23.10)		0.9571 (0.1414-7.119)	
Tumor localization[1]								
Upper third	0.1153 (0.03136-14.69)	0.4079	1.589 (0.3918- 6.284)	0.0737	0.8255 (0.2630-7.917)	0.1525	0.3964 (0.1414-7.119)	0.3510
Middle third	0.8265 (0.006324-10.62)		3.01 (0.8615-11.66)		2.567 (0.4813-23.10)		1.5 (0.2261-2.809)	
Lower third	3.904 (0.07381-7.799)		9.329 (3.414-15.07)		4.199 (0.7220-13.36)		2.211 (1.613-2.552)	
Histological grade, differentiation								
Well	NA	0.4902	NA	0.9767	NA	0.3661	NA	0.8610
Moderate	0.8039 (0.03136-14.69)		3.031 (0.3918-15.07)		1.454 (0.2630-23.10)		0.9571 (0.1414-7.119)	
Poor	0.2908 (0.006324-1.626)		2.6 (1.826-4.993)		2.514 (1.057-18.57)		1.5 (0.5035-2.532)	
Tumor stage								
1 + 2	0.5728 (0.03349-10.62)	0.9599	2.505 (1-15.07)	0.778	1.454 (0.4813-20.53)	0.7780	1.832 (0.1414-2.809)	0.4457
3 + 4	0.8039 (0.006324-7.799)		3.01 (0.4633-6.566)		3.167 (0.6082-23.10)		0.9571 (0.2261-2.532)	

[1]The analyses were performed considering only the upper and middle thirds because the number of individuals with ESCC in the lower third was too small. Statistical analyses were performed using Mann-Whitney or Kruskal Wallis test and Dunn's post-test, as appropriate; no significant differences were observed. NA: Not applicable because the number of individuals was too small.



Area	0.8240
Std. Error	0.06382
95%CI	0.6988 to 0.9491
P value	0.0001697

Figure 3 Receiver operating characteristic curve for the discrimination of healthy esophagus and normal-appearing surrounding tissue of esophageal squamous cell carcinoma patients, according to *CHRNB4* expression. The receiver operating characteristic curve was performed for *CHRNB4* mRNA expression normalized with *GAPDH* (number of healthy individuals: 32, number of esophageal squamous cell carcinoma patients: 19). For a *CHRNB4* expression cut-off of 1.429 × 10^{-5}, the area under the curve was 0.824, with a sensitivity of 75.86% and a specificity of 78.95%, *P* = 0.0002.

The sensitivity rate was 75.86% and the specificity rate was 78.95% (*P* = 0.0002, ROC curve) (Figure 3).

Prediction of survival of ESCC patients by CHRNA5 expression

Finally, we evaluated the impact of *CHRNA5* and *CHRNA7* expression on overall survival. Multivariate analysis identified *CHRNA5* expression as an independent prognostic factor of ESCC. ESCC patients with high *CHRNA5* expression showed an increased overall survival, in comparison with the ESCC patients who had low expression (Figure 4). The corresponding age- and tumor stage-adjusted hazard ratio was 0.2684 (95%CI: 0.075-0.97, *P* = 0.045).

DISCUSSION

Expression of CHRNs in extra-neuronal tissues has been extensively reported[25]; however, to date, only one study has suggested the existence of a functional non-neuronal cholinergic system, present in the human esophageal epithelium. Nguyen and colleagues[26] showed that the human esophagus expresses the enzymes responsible for choline synthesis and degradation [*i.e.,* choline acetyltransferase and acetylcholinesterase (AChE)], as well as four CHRN subunits (CHRNA3, CHRNA5, CHRNA7 and CHRNB2, which were evaluated in this study). The data in our current study agree with these previous findings; specifically, we were able to detect the mRNA of the same alpha subunits in the esophageal mucosa from

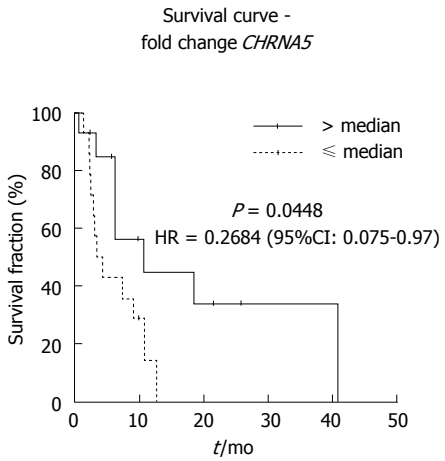


Figure 4 Kaplan-Meier curve showing the impact of *CHRNA5* mRNA expression on the overall survival of esophageal squamous cell carcinoma patients. Kaplan-Meier survival curve according to *CHRNA5* expression fold-change (ratio between expression in the tumor and the normal surrounding tissue), with follow-up duration of 5 years after diagnosis (total *n* = 28). Censored values (+) indicate the last known follow-up time for the corresponding subjects. Adjustments were performed for tumor stage and age.

healthy individuals. Furthermore, we also showed-for the first time-the presence of *CHRNB4* in this epithelium; however, the mRNA of *CHRNA1*, *CHRNA4, CHRNA9* and *CHRNA10* were undetectable.

Following the confirmation of the expression of *CHRNs* in the human esophagus, we investigated their expression in ESCC samples and matched normal-appearing surrounding mucosa. Similar to the previous findings for lung cancer[27], we observed a statistically significant overexpression of *CHRNA5* and *CHRNA7* in tumors, as compared with expression in the matched adjacent tissue and in esophageal mucosa from healthy individuals. These findings suggest a role for these receptors in the pathogenesis of epithelial tumors, especially for tumors related to tobacco smoking. However, different from the previous findings reported for lung squamous cell carcinoma[27], the overexpression of these subunits in ESCC is probably not induced by tobacco components since there was no association found between the overexpression of these receptors and the smoking status of the ESCC patients in our study.

Whereas *CHRNA5* and *CHRNA7* overexpression seems to follow esophageal transformation, the induction of *CHRNB4* expression seems to occur before the first histopathological alterations associated with development of ESCC. We showed that although there is no difference in the *CHRNB4* expression of tumor samples and the matched surrounding mucosa samples of ESCC patients, both tissues present a significantly higher *CHRNB4* expression in comparison with esophagus samples from healthy individuals. Therefore, it is tempting to speculate that the consumption of high doses of alcohol and/or tobacco, characteristic of ESCC patients, could influence the expression of this receptor, affecting both tumor and

surrounding tissues. This speculation agrees with the hypothesis of field cancerization, which was proposed to explain the high propensity for development of multiple, independent tumors in the mucosal tissues of the head and neck as a consequence of risk factor exposure, a characteristic also observed in the esophagus[28]. In this context, the epithelium adjacent to the tumor may appear normal histologically but may already harbor molecular alterations, such as *CHRNB4* overexpression. Following this hypothesis, we evaluated how efficiently *CHRNB4* expression levels could distinguish the esophageal mucosa of healthy individuals from the surrounding normal-appearing epithelium of ESCC patients. Interestingly, *CHRNB4* expression was able to discriminate the tissues with 75.86% sensitivity and 78.95% specificity, suggesting its potential utility as a predictive marker of field cancerization in the esophagus; further studies are necessary to verify this hypothesis, however.

The contribution of tobacco smoking to the development of several tumor types, such as lung, head and neck, and esophagus, is a consensus. More recently, the impacts of post-diagnosis exposure to tobacco components on treatment response and survival have emerged as a hot topic. In this context, patients with tobacco smoking-related lung cancer who continue smoking have a poorer prognosis[29,30]. Tobacco is, therefore, not only a source of carcinogens responsible for tumor initiation but also of tumor promoting agents, such as nicotine, which is able to induce cell proliferation, inhibit apoptosis and induce epithelial-mesenchymal transition, to name a few of its known effects[29]. However, studies that have evaluated the impact of the cholinergic system on prognosis of patients diagnosed with tobacco-related tumors are still scarce. Yoo and colleagues[31] reported that demethylation of *CHRNB4*, which is correlated with increased mRNA expression, confers a poorer prognosis in patients with non-small cell lung cancer. Castillo-González and colleagues[32] reported that low AChE activity is correlated with poor overall survival in patients with head and neck cancers. For ESCC, the current study is the first to report an impact of alterations of the cholinergic system on patient prognosis. Specifically, *CHRNA5* expression was identified as an independent prognostic factor for ESCC, with patients who present a higher expression of this receptor showing a better overall survival. It is unclear how this overexpression could protect against tumor progression, but it has already been shown by others that under- or over-activation of the *CHRNA5* promoter is protective against lung cancer development[33], suggesting a role of this receptor in tissue homeostasis.

The current study is also the first to investigate the expression profile of *CHRNs* in both healthy esophagus and ESCC (tumor and tumor-adjacent) tissues. Although the number of samples was limited, the results show homogeneous expression of *CHRNA3*, *CHRNA5*, *CHRNA7* and *CHRNB4* along the entire esophagus under normal, non-cancerous condition and suggest that nicotine and/or alcohol exposure are not capable of affecting the expression of these receptors in the healthy mucosa. Additionally, *CHRNA1*, *CHRNA4, CHRNA9* and *CHRNA10* were not detected in the esophageal epithelium, but this lack of expression should be validated by other techniques. A similar evaluation should also be carried out for the other subunits that were not assessed in the present study due to the lack of positive controls; these unexamined receptor genes include *CHRNA2, CHRNA6, CHRNB2* and *CHRNB3*. Furthermore, this study also showed deregulation of *CHRNA5* and *CHRNA7* expression in ESCC, which may contribute to the esophageal carcinogenesis process. Finally, *CHRNB4* overexpression was shown to be an early alteration of ESCC carcinogenesis and *CHRNA5* expression as an independent prognostic factor. Such characterization provided evidence that the esophageal epithelium possesses a functional cholinergic system, which is deregulated in ESCC, but further analyses are now necessary to better comprehend which pathways could be affected by this deregulation and how this could contribute to the progression of esophageal cancer.

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COMMENTS

Background

Esophageal squamous cell carcinoma (ESCC) is one of the most incident and lethal tumors worldwide. Although tobacco and alcohol are recognized as the main risk factors of the disease, the molecular mechanisms involved in its development remain unclear. Tobacco components are well recognized for their ability to induce mutations and to activate cellular pathways correlated with tumor progression. In this context, the nicotinic cholinergic receptors (CHRNs) may play a central role in ESCC, but data regarding their expression in the esophagus, both under normal and pathologic conditions, is still very limited.

Research frontiers

So far, only one study in the publicly available literature has suggested the existence of a functional cholinergic system in normal esophageal epithelium and the expression of CHRNs have not been evaluated in ESCC tissues.

Innovations and breakthroughs

This is the first study to show that *CHRNA3*, *CHRNA5*, *CHRNA7* and *CHRNB4* are homogeneously expressed in the esophageal mucosa of healthy individuals. Moreover, the expression of these *CHRNs* does not seem to be modulated by tobacco and/or alcohol exposure. We also show, for the first time, overexpression of *CHRNA7* and *CHRNA5* in ESCC, with the latter showing an impact on prognosis. Finally, the findings from our study support the possibility that *CHRNB4* overexpression is an early alteration during esophageal carcinogenesis, preceding the first histopathological alterations.

Applications

Identifying the molecular alterations that take place during esophageal carcinogenesis may help not only to elucidate which mechanisms contribute to ESCC development and progression but may also identify new biomarkers of diagnosis and prognosis. This knowledge is of utmost relevance for improving overall survival of ESCC patients.

Terminology

Nicotinic CHRNs are recognized as important proteins that mediate chemical neurotransmission at neurons, ganglia, interneurons and the motor end plate. However, the ubiquitous expression of CHRNs in mammalian cells has suggested they may play an additional role in extra-neuronal tissues. In fact, different studies have shown their participation in maintaining communication and phenotypic functions of non-neuronal cells and the deregulation of these receptors has been observed in different tumor types. CHRN activation by tobacco components triggers different cellular pathways involved in survival and apoptosis blockade and may contribute to tumor progression by these mechanisms.

Peer-review

The study shows expression of CHRN subunits α3, α5, α7 and β4, but not α1, α4, α9 and α10, in normal esophageal mucosa. In ESCC, CHRNA5 and CHRNA7 subunits were found overexpressed when compared to matched surrounding mucosa. CHRNB4 was differentially expressed between healthy esophagus and normal-appearing ESCC adjacent mucosa. *CHRNA5* expression is an independent prognostic factor in ESCC. Patients with high *CHRNA5* expression showed an increased overall survival in comparison with those with low expression.

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