IJBM The International Journal of Biological Markers

Original research article

β-Arrestin-1 expression and epithelial-to-mesenchymal transition in laryngeal carcinoma

The International Journal of Biological Markers
2019, Vol. 34(1) 33–40

DOI: 10.1177/1724600818813621
journals.sagepub.com/home/jbm
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Abstract

Aim: The novel primary end-point of the present study was to ascertain β-arrestin-1 expression in a cohort of consecutive patients with laryngeal squamous cell carcinoma (LSCC) with information available on their cigarette-smoking habits. A secondary end-point was to conduct a preliminary clinical and pathological investigation into the possible role of β-arrestin-1 in the epithelial-to-mesenchymal transition (EMT), identified by testing for E-cadherin, Zeb1, and Zeb2 expression, in the setting of LSCC.

Methods: The expression of β-arrestin-1, E-cadherin, zeb1, and zeb2 was ascertained in 20 consecutive LSCCs.

Results: Statistical analysis showed no significant associations between β-arrestin-1 and EMT (based on the expression of E-cadherin, Zeb1, and Zeb2). The combined effect of nicotine and β-arrestin-1 was significantly associated with a shorter disease-free survival (P=0.01) in our series of LSCC. This latter result was also confirmed in an independent, publicly available LSCC cohort (P=0.047).

Conclusions: Further investigations on larger series (ideally in prospective settings) are needed before we can consider closer follow-up protocols and/or more aggressive treatments for patients with LSCC and a combination of nicotine exposure and β-arrestin-1 positivity in tumor cells at the time of their diagnosis. Further studies on how β-arrestin functions in cancer via different signaling pathways might reveal potential targets for the treatment of even advanced laryngeal malignancies.

Keywords

Laryngeal carcinoma, β-arrestin-1, epithelial-to-mesenchymal transition, nicotine, prognosis

Date received: 4 June 2018; revised: 1 October 2018; accepted: 19 October 2018

Introduction

Laryngeal squamous cell carcinoma (LSCC) is the fifteenth most common human malignancy in the USA according to the Surveillance, Epidemiology, and End Results Cancer Statistics Review (1975–2008), and the twenty-first for both genders in the European Union (of 27 Member States) according to the European Cancer Observatory data (2008).[1] Tobacco smoke is the main risk factor implicated in laryngeal carcinogenesis.[2-4]

Tobacco smoke contains numerous classes of carcinogens, such as polycyclic aromatic hydrocarbons, tobacco-specific nitrosamines, and aldehydes. These elements are

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capable of initiating tumorigenesis primarily through the formation of DNA adducts, resulting in mutations of such vital genes as KRAS, p53, and Rb.[5] Nicotine, the main addictive component of tobacco smoke, seems not able to initiate tumorigenesis in humans, but it has been found to induce proliferation, invasion, and epithelial-to-mesenchymal transition (EMT) in non-small-cell lung cancer cell lines. Nicotine prompts changes in cell morphology and ablates tight junctions—a situation consistent with EMT, but β-arrestin-1 is required for these changes to take place.[6] The binding of nicotine to nicotinic acetylcholine receptors (nAChR) leads to the formation of an oligomeric complex between nicotinic acetylcholine receptor, SRC, and β-arrestin-1, which seems to be vital to the nicotine-induced proliferation of human non-small cell lung cancers.[7] Arrestins form a small family of proteins with four isoforms in humans.[8] β-arrestins are ubiquitously expressed proteins first described for their role in desensitizing G-protein-coupled receptors (GPCRs).[9] Many pools of arrestins are essential to the control of cell metabolism, division, motility, and cross-talk, and are adapted to provide diverse, but highly specific signal integration.[10] β-arrestin-1 has been shown to promote several stages in the progression of cancer, including invasion and metastasis in cancers of the breast, ovary, and colon.[7]

EMT is a complex process in which epithelial cells lose their typical features and acquire a mesenchymal-like phenotype, which enhances their ability to migrate. This takes place in a number of stages that lead to epithelial cells losing their cell adhesiveness and apical–basal polarity, and undergoing both cytoskeletal and signaling changes that confer invasive properties.[11] These events involve a molecular cell reprogramming that entails E-cadherin levels[12] being downregulated by nuclear factors such as Snail, Slug, Twist, zinc finger E-box binding homeobox 1 (Zeb1), and zinc finger E-box binding homeobox 2 (Zeb2).[13]

The novel primary end-point of the present study was to test for β-arrestin-1 expression in a cohort of consecutive patients with LSCC with information available on their cigarette-smoking habits. A secondary end-point was to conduct a preliminary clinical and pathological investigation into the possible role of β-arrestin-1 in EMT, identified on the basis of E-cadherin, Zeb1, and Zeb2 levels, in the setting of LSCC.

Methods

Patients

The study was conducted in accordance with the principles of the Helsinki Declaration. Data were examined in agreement with the Italian privacy and sensible data laws (D. Lgs 196/03) and the Otolaryngology Section's University of Padova internal rules. Before undergoing surgery, all patients included in the study signed a detailed informed consent form.

From the archives at the Pathology Department of Padova University, we retrieved 20 consecutive patients with LSCC (15 males and 5 females; mean age 62.4±8.0 years) treated with primary surgery, for whom information was available about their cigarette-smoking habits, as well as their clinical details. As in the recommendations adopted for LSCC at our institution,[14,15] all patients had undergone microlaryngoscopy with laryngeal biopsy, upper aero-digestive tract endoscopy, neck ultrasonography (with or without fine-needle aspiration cytology), head and neck contrast-enhanced computed tomography (CT), and/or magnetic resonance imaging, chest x-ray, and liver ultrasonography.

All patients underwent laryngeal surgery at the Otolaryngology Section of the Padova University, with unilateral or bilateral cervical lymph node dissection in 18 cases. Pathological staging, and the characteristics of primaries and metastases prompted postoperative radiotherapy in five cases in accordance with current guidelines, as described elsewhere.[16] Table 1 provides details of patients' clinical and pathological features, based also on the 7th edition of the TNM Classification of Malignant Tumors.[17] No distant metastases (M) were detected at diagnosis. As previously reported,[15] the scheduled clinical follow-up after treatment (adjustable to patients' individual characteristics) adopted at our institution was: (a) once a month in the first year; (b) every 2 months in the second year; (c) every 3 months in the third year; (d) every 4 months in the fourth year; (e) every 6 months in the fifth year; and (f) every 12 months thereafter. Neck ultrasonography and chest x-rays were also performed at least annually. Contrast-enhanced CT of the neck, total body positron emission tomography, chest CT, and liver ultrasonography were repeated as necessary. The mean follow-up was 42.2±31.2 months.

Immunohistochemistry

Immunohistochemical studies were conducted on formalin-fixed, paraffin-embedded sections 4 to 5 μm thick obtained from each tissue sample. Staining was done automatically using a fully automated system (Bond™-maX; Leica, Newcastle Upon Tyne, UK). Sections were pretreated using heat-mediated antigen retrieval with sodium citrate buffer (pH6, Epitope Retrieval Solution 1, Leica) for 30 minutes at 99°C. Specimens were then incubated with the panel of primary antibodies listed in Table 2, according to the manufacturer's instructions. Sections were then lightly counterstained with hematoxylin. Appropriate positive and negative controls were run concurrently. Immunoreactions for β-arrestin-1 were detectable in the cytoplasm, those for E-cadherin and Zeb1/Zeb2 in the cellular membrane and nucleus, respectively. Immunohistochemical reactions were scored semiquantitatively by two pathologists (R.C. and L.N.) on a

Table 1. β-Arrestin-1 immunohistochemical staining in LSCCs stratified by patients' demographics, and by clinical and pathological variables.

	Negative for arrestin (14 cases)	Positive for arrestin (6 cases)	*P*-value
Age (years)	60.8 ± 8.5	66.2 ± 6.9	0.406*
Smoking habits (No., %)			0.187**
− smoker	7 (50%)	5 (83.3%)	
− non-smoker or former smoker	7 (50%)	1 (16.7%)	
LSCC grade (No., %)	(2 missing)	(1 missing)	0.205**
− 1–2	9 (75%)	2 (40%)	
− 3	3 (25%)	3 (60%)	
Pathological stage (No., %)			0.723**
− I–II	9 (64.3%)	4 (66.7%)	
− III–IV	5 (35.7%)	2 (33.3%)	
Postoperative radiotherapy (No., %)			1**
− not performed	10 (71.4%)	5 (83.3%)	
− performed	4 (35.7%)	1 (16.7%)	
E-cadherin staining (No., %)			0.657**
− negative	3 (21.4%)	1 (16.7%)	
− positive	11 (78.6%)	5 (83.3%)	
Zeb1/Zeb2 staining (No., %)			0.336**
− negative	10 (71.4%)	3 (50%)	
− positive	4 (35.7%)	3 (50%)	

IHC: immunohistochemical; LSCC: laryngeal squamous cell carcinoma.
*Wilcoxon's rank-sum test.
**Fisher's exact test.

Table 2. Panel of primary antibodies.

Antigen	Clone	Source	Vendor	Dilution
β-arrestin-1	Ab32099	Rabbit monoclonal	Abcam	1:200
E-cadherin	Clone NCH38	Mouse monoclonal	DakoCytomation, Glostrup, Denmark	1:200
Zeb1	sc-25388	Rabbit polyclonal	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:100
Zeb2	sc-271984	Mouse monoclonal	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:100

two-tiered scale, based on the percentage of positive tumor cells, as: negative (0–30%); or positive (>30%).

Statistical analysis

Between-group statistical comparisons were drawn to test whether there were either clinical or immunohistochemical factors significantly associated with β-arrestin-1 positivity (categorical: negative vs. positive scores). Specifically, the two-sided Wilcoxon rank-sum test was used for continuous variables, and Fisher's exact test for categorical variables. The following factors were considered: age, smoking habits (categorical: smokers vs. non-smokers or former smokers), tumor histological grading (categorical: 1–2 vs. 3), tumor pathological staging (categorical: I–II vs. III–IV), postoperative radiotherapy (categorical: performed vs. not performed), E-cadherin and Zeb1/Zeb2 immunohistochemical scores (categorical: negative vs. positive scores).

A test was then run to see if smoking and/or β-arrestin-1 positivity, separately or in combination, could significantly alter survival probability. The Kaplan–Meier method and the log-rank test were used, considering changes within 2 years of disease-free survival. To test for their combined effect, smokers who were positive for β-arrestin-1 were compared with all the other patients, assuming that only the concomitant presence of nicotine and β-arrestin-1 could induce a worse prognosis in LSCC. Cox's regression analysis was used to retrieve the proportional hazard ratios for the survival data associated with the effect of the combination of nicotine and β-arrestin-1 positivity. The above-mentioned clinical and immunohistochemical factors were initially included as confounders in the full model, considering only the main effects. They were then removed sequentially if this did not prompt in a significant change in the estimates, using the Likelihood Ratio test to compare the models with and without the inclusion of each confounder.

For all the statistical tests, a P-value <0.05 was considered significant. All the statistical analyses were performed with the R package, version 3.4.3.

Validation on public gene expression data

The significant results obtained from the statistical analysis in our LSCC cohort were further tested on an independent publicly available gene expression dataset. A total of 48 LSCC patients were considered from a cohort of 290 head and neck squamous cell carcinoma patients reported in the study by Wichmann et al.,[18] based on HPV16 DNA and RNA status, gene expression patterns, and mutated candidate genes. From this cohort, 36 LSCC patients with primary non-metastatic tumors and available information on both progression-free survival and smoking history (six non-smokers) were selected. Gene expression of β-arrestin-1 was retrieved from the preprocessed and normalized data available in ArrayExpress (accession number: E-GEOD-65858), and high/low expression levels were defined according to the average expression value.

Results

Patients' clinical outcomes

Fifteen of the 20 patients with LSCC experienced no disease recurrence after treatment, while 5 relapsed after a mean 9.8 ± 2.3 months.

LSCC: β -arrestin-1 expression, clinical and pathological features, and prognosis

Immunohistochemical analyses were performed on our series of 20 LSCC samples. Immunostaining showed that 6 of the 20 patients (30%) were positive for β-arrestin-1. Diffuse staining for β-arrestin-1 was seen in both the cytoplasm and in some nuclei of the cancer cells, while the immune and stromal cells were negative (Figure 1).

Between-group comparisons revealed no statistically significant associations between β-arrestin-1 positivity and patients' clinical and pathological factors ($P>0.1$) (Table 1). Survival analysis showed no significant difference in prognosis when either nicotine or β-arrestin-1 positivity was considered separately (log-rank test, $P=$ 0.29 and 0.06, respectively), but in combination they were significantly associated with a shorter disease-free survival ($P=0.01$, Figure 2(a)). The survival model was unaffected by the inclusion of additional confounding factors, whose coefficients were not statistically significant ($P>0.2$). This result was also confirmed in the LSCC cohort published by Wichmann et al.[18] ($P=0.76$ for nicotine; $P=0.07$ for β-arrestin-1 positivity; $P=0.047$ for the combination; Figure 2(b)).

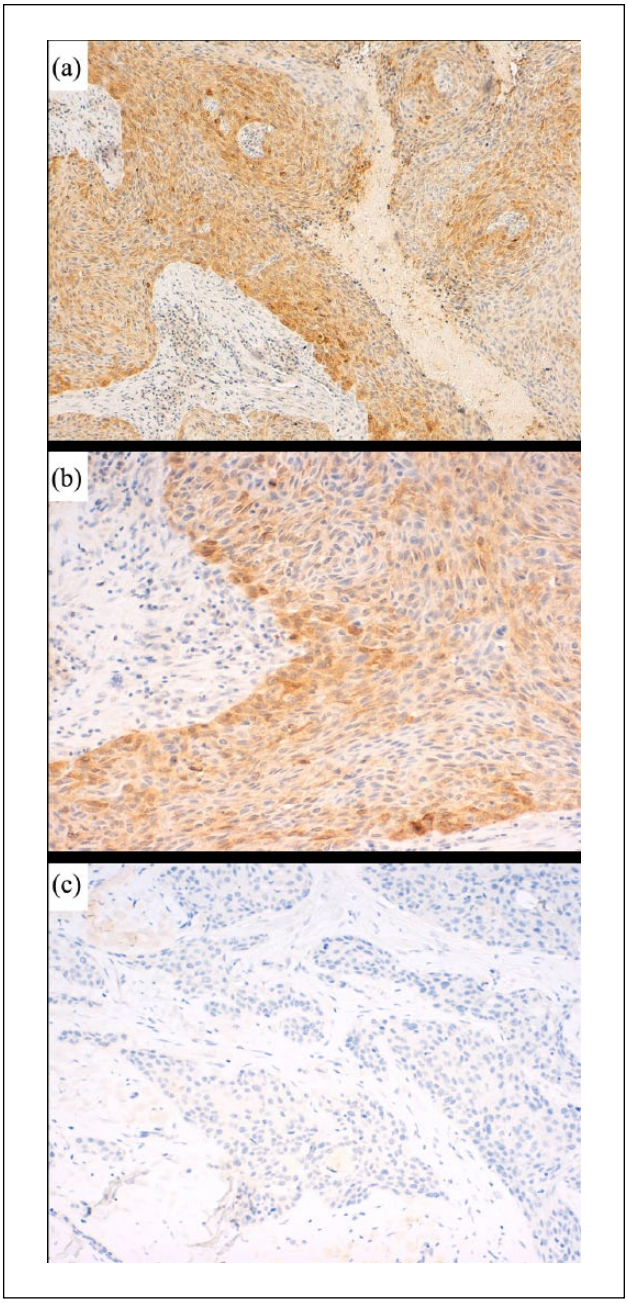

Figure 1. Representative cases of positive staining for β-arrestin-1 (a) and (b): all positive cases showed a homogeneous pattern with diffuse cytoplasmic staining and (as shown in (b)) occasional positive nuclei. Representative case of negative staining for β-arrestin-1 (c). Original magnification 100X for (a) and 200X for (b) and (c).

Association between β -arrestin -1 expression and biomarkers of EMT in LSCC

To test the association between β-arrestin-1 and EMT, immunohistochemical analyses were performed to assess the expression of E-cadherin, Zeb1, and Zeb2 in tumor cells. E-cadherin positivity emerged in 16 cases (80%),

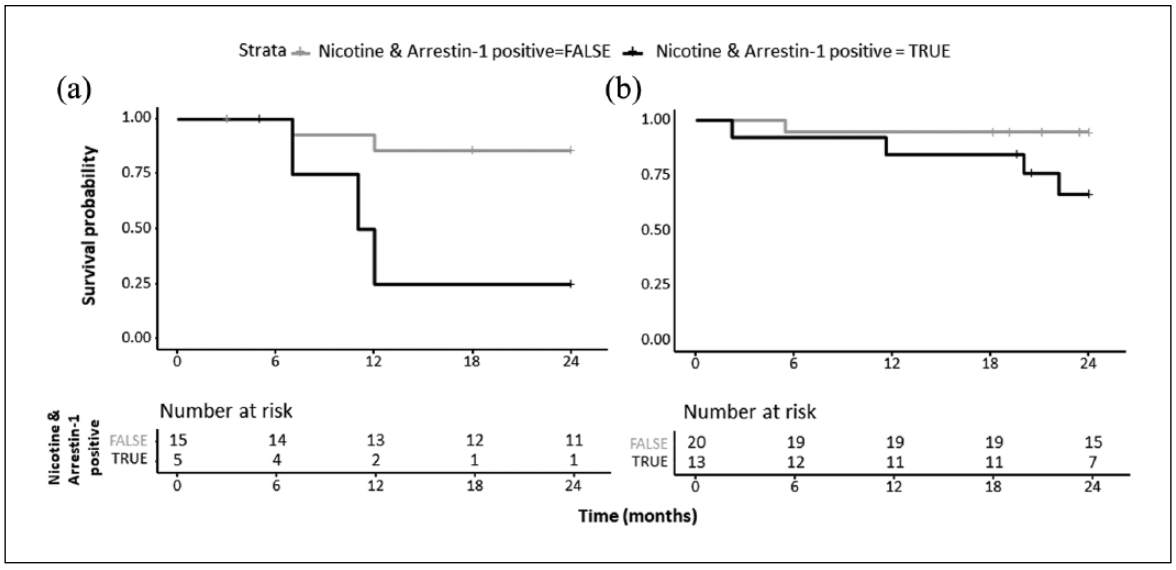

Figure 2. Kaplan–Meier curves for disease-free survival in LSCC, considering the combined effect of nicotine and β-arrestin-1 positivity; time intervals in months. (a) Results from Padova's LSCC cohort. (b) Results from the public LSCC cohort.[18] LSCC: laryngeal squamous cell carcinoma.

which showed diffuse membranous and cytoplasmic staining. Zeb1 was positive in two cases (10%), with nuclear staining. Zeb2 was positive in six cases (30%), again with nuclear staining (Figure 3). Statistical analysis revealed no significant associations between β-arrestin-1 and EMT.

Discussion

While nicotine is unable to initiate tumorigenesis in humans, it has been shown to promote tumor growth and metastasis by inducing cell cycle progression, migration, invasion, angiogenesis, and evasion of apoptosis in a variety of systems.[5] Nicotine can also induce changes in gene expression consistent with EMT, a signature of more advanced and less differentiated cancers. The EMT program is a highly conserved developmental event that promotes epithelial cell dissociation and migration to appropriate sites during embryogenesis. In malignancies, this very same mechanism also facilitates the metastatic cascade. β-Arrestin-1 upregulates EMT, promoting transcription factors such as Zeb1 and Zeb2.[6] A novel role of β-arrestin-1 in modulating EMT and glycogen synthase kinase 3 β/β-catenin signaling has recently been hypothesized in prostate cancer.[19]

The EMT program was recently investigated in LSCC too.[20] In 2015, Cappellesso et al.[12] analyzed the clinical and pathological role of E-cadherin, N-cadherin, Snail, Slug, Zeb1, and Zeb2 in 37 consecutive cases of LSCC. Low E-cadherin levels and high Slug levels correlated with both a higher disease recurrence rate and a shorter disease-free survival. The relative levels of CDH1, SNAI2, miR-1, and the miR-200 family were also examined, and a significant association with disease recurrence was found for CDH1, miR-200a and miR-200c downregulation, and for SNAI2 overexpression. Greco et al.[21] investigated the clinical significance of E-cadherin, N-cadherin, β-catenin, α-catenin, γ-catenin, caveolin-1, and vimentin in a cohort of 82 patients with LSCC treated surgically, with or without adjuvant therapy. They found that cytoplasmic β-catenin overexpression was independently associated with a longer disease-specific survival, and E-cadherin overexpression emerged as an independent risk factor for poor overall survival (OS). Zuo et al.[22] considered 57 surgical samples from patients with laryngeal cancer. They found that the expression of glucose metabolism, such as glucose transporter-1 (GLUT-1), correlated positively with the expression of vimentin and N-cadherin in LSCC tissues, but negatively with the expression of E-cadherin. The survival rate of patients who were found positive for GLUT-1, vimentin, and N-cadherin was significantly shorter than for those who were not. The authors concluded that hypoxia promoted laryngeal cancer cell invasion and migration via EMT. Recently, Zhu et al.[23] used immunohistochemistry to investigate E-cadherin, N-cadherin, β-catenin, and Zeb2 protein expression in a cohort of 76 patients with operable LSCC. They found negativity for E-cadherin expression and positivity for N-cadherin, β-catenin and Zeb2 expression associated with a shorter OS. Multivariate analysis indicated that T stage, β-catenin, and Zeb2 expressions were independent risk factors for OS in LSCC.

The present clinical and pathological study is the first to investigate the possible role of β-arrestin-1 in EMT in the setting of LSCC by testing for the tissue expression of

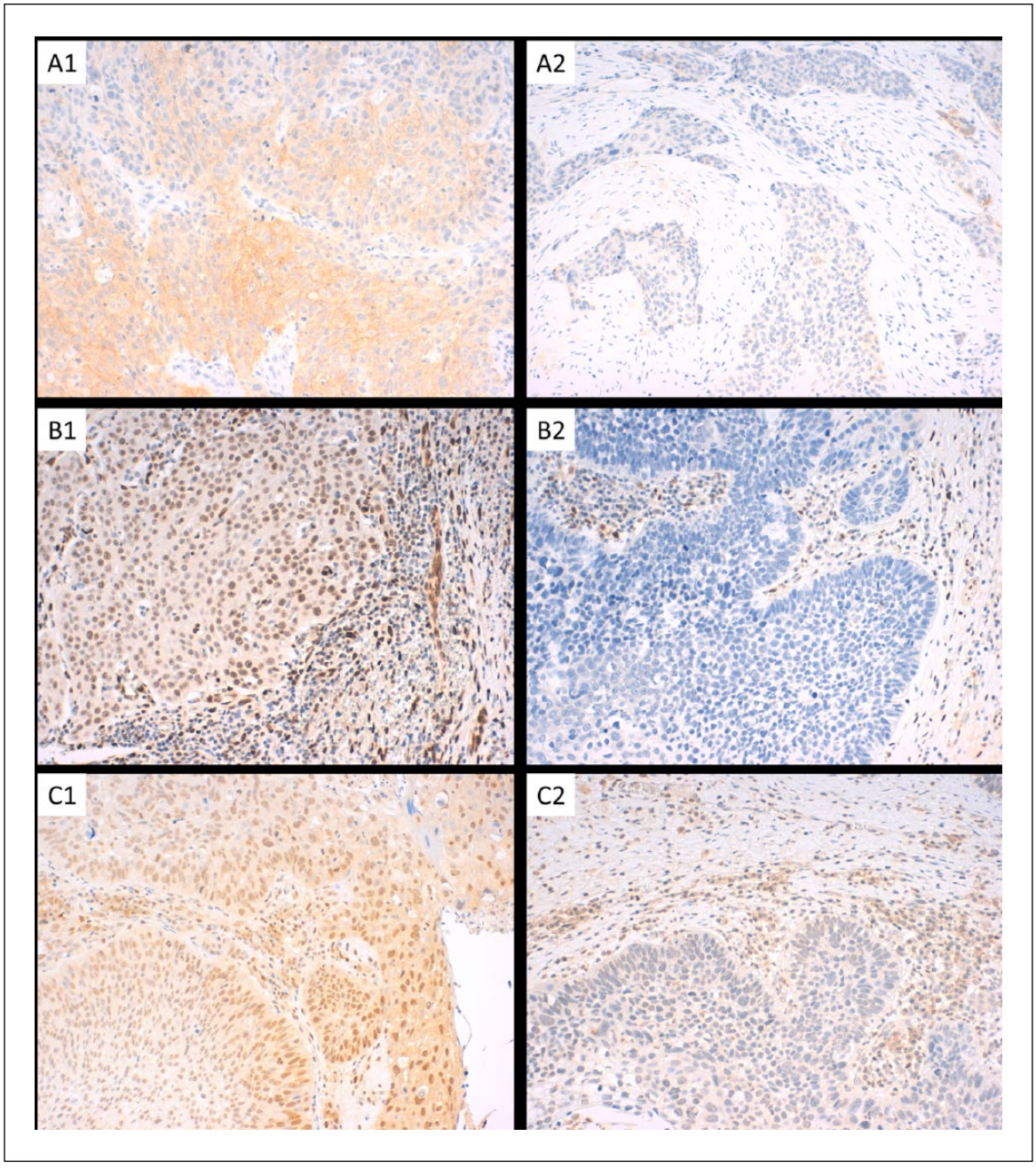

Figure 3. Representative cases of positivity for EMT-related genes E-cadherin, Zeb1, and Zeb2. In particular, a positive case (A1) and a negative case (A2) for E-cadherin; a positive (B1) and a negative (B2) case for Zeb1; and a positive (C1) and a negative (C2) case for Zeb2 (original magnification 200X). EMT: epithelial-to-mesenchymal transition.

some crucial biomarkers, such as E-cadherin, Zeb1, and Zeb2. The main strength of this study lies in the homogeneity of the series of patients considered, since (a) they all underwent primary laryngeal surgery; (b) their surgical treatment was managed by the same team; (c) a standardized follow-up protocol was implemented for all patients; (d) only squamous cell carcinomas located in a single structure of the upper aerodigestive tract (the larynx) were considered; and (e) only surgical specimens (not biopsies) of LSCC were assessed. The main weaknesses of this study, on the other hand, concern its retrospective setting and the limited number of cases considered.

Our statistical analysis revealed no significant associations between β-arrestin-1 and EMT, as identified by testing for E-cadherin, Zeb1, and Zeb2. Survival analysis indicated no significant prognostic differences when nicotine and β-arrestin-1 were considered separately. On the other hand, our results and their validation on an

independent cohort provide preliminary support for the hypothesis that a combination of nicotine and β-arrestin-1 positivity might be significantly associated with a shorter disease-free survival in surgically treated LSCC. Fox et al.[24] reported that lung cancer patients who continued to smoke had a shorter median survival than non-smokers and former smokers, particularly when diagnosed with earlier-stage (I–II) lung cancer. Since disease recurrence in early-stage lung cancer patients is associated with a poor survival, it may be that effects of nicotine and its receptors on tumor cells contribute to a worse outcome in smokers.[25] Perumal et al.[25] identified genes that are differentially regulated by nicotine in an ARRB1/β-arrestin-1-dependent manner in human non-small-cell lung cancer cells. Among the genes they identified, stem cell factors strongly differentiated smokers from non-smokers, and its strong expression correlated with a poor prognosis.

However, our cohort does not provide enough cases for all the combination of factors to robustly assess the statistical impact of the combined effect of nicotine and β-arrestin-1 in disease-free survival. Specifically, out of six patients who were β-arrestin-1 positive, only two survived disease free at 2 years, including the only non-smoker in this group, versus 18 among 20 β-arrestin-1 negative patients (Figure 2). In addition, the validation dataset from Wichmann et al.[18] shows a limited number of non-smokers (only six) and only one of them with high β-arrestin-1 gene expression. As a possible validation dataset, we have also considered the 79 LSCC patients available from the cohort of 279 head and neck squamous cell carcinoma patients reported by The Cancer Genome Atlas.[26] Only 46 cases have gene expression data with available information on disease-free survival, and all these patients are smokers, which makes the comparison with the disease-free survival analysis from our study unfeasible. After looking at the main databases for gene expression data (i.e. Gene Expression Omnibus and ArrayExpress), to the best of our knowledge there are no other LSCC cohorts providing mRNA expression data with available information on both disease-free survival and smoking history for the patients.

Further investigations on larger series, ideally in prospective settings, are needed before we can say whether the above-reported evidence would support closer follow-up protocols and/or more aggressive treatments for patients with LSCC and a combination of nicotine exposure and β-arrestin-1 positivity in tumor cells at diagnosis. Moreover, further studies should assess the real activity of β-arrestin-1 extending expression studies to β-arrestin-1 substrates as the phosphorylated Src protein, which is a downstream substrate of activated β-arrestin-1, and in turn orchestrate the entire EMT transcriptomic machinery.[27] Efforts in the field of targeted therapeutics for human cancers have begun to find novel targets for pharmacological treatments. β-arrestin-1 is a well-known primary effector of the GPCR pathway, which is crucial to tumor growth and progression.[28] A variety of β-arrestin-1-biased ligands, which readily associate with β-arrestin-1, have been identified, including nicotinic acetylcholine receptors, EP2- and EP4-receptors, the endothelin-A receptor, and transforming growth factor β.[29,30] Biased ligands are capable of specifically altering the conformation of a receptor, whereas a specific receptor conformation cannot activate all of its downstream signals in parallel; it can only promote a particular downstream signal.[30] Further studies on how β-arrestins-1 function in cancer via different signaling pathways could point to a potential target for the treatment of even advanced laryngeal malignancies.

Acknowledgements

The authors thank Frances Coburn for correcting the English version of this paper.

Declaration of conflicting interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was partly supported by grant No. DOR 1658072/16 (G. Marioni) from the University of Padova, Italy.

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