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ORIGINAL ARTICLE

Beta1- and Beta2-Adrenoceptors Expression Patterns in Human Non-small Cell Lung Cancer: Relationship with Cancer Histology

Marisa Coelho[1,2,3] · Andrea Imperatori[4] · Anna Maria Chiaravalli[5] · Francesca Franzi[5] · Massimo Castiglioni[4] · Emanuela Rasini[3] · Alessandra Luini[3] · Massimiliano Legnaro[3] · Franca Marino[3] · Laura Ribeiro[1,2] · Marco Cosentino[3]

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Abstract

Assessment of Beta-AR protein expression on tumour tissues might be a plausible strategy to select cancer patients who can benefit from Beta-blockers therapy. The aim of this study is to evaluate the differences between resected tissue specimens from primary lung cancer (adenocarcinoma (ADC) and squamous cell carcinoma (SCC)) in terms of expression pattern of Beta1- and Beta2-AR in both tumour and adjacent surrounding non-tumour tissue. This retrospective study was based on the analysis of 80 patients with histologically confirmed diagnosis of primary Non-Small Cell Lung Cancer (NSCLC) who received surgical treatment. The cases were carefully selected in order to obtain the most homogeneous sample in terms of histologic subtype (40 ADCs and 40 SCCs) and clinical stage (10 each). Beta1- and Beta2-AR expression was determined by immunohistochemistry and the staining evaluated by semi-quantitative scoring using the H-score method. In our NSCLC series, Beta1- and Beta2-AR are differentially expressed. Beta1-AR expression is present at low levels in both SCC and ADC. Likewise, when compared with the matched surrounding non-tumour tissues, Beta1-AR expression level was significantly lower in both histologic subtypes. Conversely, Beta2-AR is highly expressed in both histologic subtypes, but clearly highly expressed in ADC when compared with SCC and with their matched surrounding non-tumour tissue. Overall, this clinicopathological study highlights the differential expression of Beta1- and Beta2-AR in ADC and SCC. Repurposing non-selective Beta-blockers in oncologic setting might be a suitable therapeutic strategy for lung ADC.

Keywords Beta-adrenoceptors · Non-small cell lung cancer · Clinicopathological study · Immunohistochemistry

Introduction

Primary lung cancer is one of the most frequently diagnosed malignancy and the leading cause of cancer-related death worldwide (Yang 2009). Non-small cell lung cancer (NSCLC) accounts for about 85% of the total number of lung cancer cases and represents a heterogeneous disease that is further divided into three main categories: adenocarcinoma (ADC), squamous cell carcinoma (SCC) and large cell carcinoma (Novello et al. 2016). ADC and SCC are the most common NSCLC subtypes accounting respectively for roughly 40% and 30% of the total number of cases. Despite efforts in research and clinical care have advanced significantly during the last decade, the prognosis of NSCLC is still very poor (Yang 2009). The histologic subtype per se is not an independent prognostic factor for NSCLC and hence, classification named TNM, based on tumour size (T) and on nodal (N) and metastatic (M)

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✉ Marisa Coelho
mcoelho@med.up.pt

[1] Department of Biomedicine, Unit of Biochemistry, Faculty of Medicine, University of Porto, Rua Dr. Plácido da Costa, s/n, 4200-450 Porto, Portugal

[2] I3S - Instituto de Investigação e Inovação em Saúde, University of Porto, Rua Alfredo Allen, 4200-135 Porto, Portugal

[3] Center of Research in Medical Pharmacology, University of Insubria, Via Monte Generoso 71, 21100 Varese, Italy

[4] Center for Thoracic Surgery, Department of Medicine and Surgery, ASST Sette Laghi, University of Insubria, via Guicciardini 9, 21100 Varese, Italy

[5] Unit of Pathology, Department of Medicine and Surgery, ASST Sette Laghi, University of Insubria, via Rossi 9, 21100 Varese, Italy

involvement is still the most reproducible prognostic factor for this disease (Novello et al. 2016). Given the lack of more precise prognostic biomarkers in NSCLC, which is problematic considering that the appropriate therapeutic approach is based on correct identification of its histologic subtypes, it is urgent to point out new ways of research to better understand its progression and consequently improve its outcome.

Over the last years, preclinical studies have associated the activation of Beta-adrenoceptors (Beta-AR) signalling pathways with cancer progression in several types of tumours. Beta-AR belong to the superfamily of G protein-coupled-receptors (GPCR) and are classified into three different subtypes (Beta1-, Beta2- and Beta3-AR) showing distinct tissue distribution and pattern of expression. They are involved in the regulation of many important physiological functions and thus are very well known drug targets in different therapeutic areas (Cole et al. 2015; Coelho et al. 2017). Stress neurotransmitters, such as adrenaline and noradrenaline, by activation of Beta-AR contribute to the regulation of some pro-tumorigenic pathways enhancing tumour cell proliferation, migration, invasion and angiogenesis (Schuller 2010; Cole and Sood 2012; Coelho et al. 2017). In fact, Beta-AR overexpression, particularly Beta2-AR, has been reported in diverse human tumours, including melanoma (Yazawa et al. 2016b), prostate (Ramberg et al. 2008), hepatocellular (Chen et al. 2012), gastric (Takahashi et al. 2016), oral (Bravo-Calderón et al. 2012) and breast (Liu et al. 2015). Tumour-associated Beta2-AR expression was also found to be highly correlated with poor clinicopathological features, namely, tumour recurrence, metastasis, and reduced survival in the majority of the aforementioned tumours (Ramberg et al. 2008; Bravo-Calderón et al. 2012; Chen et al. 2012; Liu et al. 2015; Takahashi et al. 2016; Yazawa et al. 2016b).

In lung, Beta2-AR are already one of the most important drug targets for several lung pathologies. For instance, Beta-AR agonists are extensively used in clinical setting as first-line therapy in asthma and chronic obstructive pulmonary disease (COPD) by promoting bronchodilator effects via Beta2-AR which are localized on airway smooth muscle cells. Aside from their presence on these cells, they are also expressed in many other cells throughout the lung namely in epithelial cells, submucosal glands, vascular epithelium, vascular smooth muscle and inflammatory cells (mast, macrophages and eosinophils) (Billington et al. 2017). When expressed on tumour cells of NSCLC, it is believed that they mediate the pro-tumorigenic effects of N′-nitrosonornicotine (NNK). NNK is one of the most powerful-carcinogen from smoke which is not only an agonist for nicotinic receptors (nAChRs) but also for Beta-AR; in addition, NNK stimulates cell proliferation under a cooperative regulation of both receptors pathways (Al-wadei et al. 2012). Interesting enough, a recent study (Nilsson et al. 2017) has also reported that in NSCLC, Beta2-AR, besides modulate some pro-tumour mechanisms, they can also mediate drug resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) (EGFR-TKI) suggesting that non-selective Beta-blockers could be also useful to reverting the drug resistance that commonly is associated with this specific type of treatment (Coelho et al. 2018)

These findings, along with epidemiologic research, have been stimulating a growing number of studies pointing out and suggesting that Beta-blockers have actually, potentiality for drug repurposing in oncology setting (Schuller 2010; Nagaraja et al. 2013; Pantziarka et al. 2016). Given the promise of Beta-blockers as a new therapeutic approach for oncological diseases, it is crucial to find strategies to identify and select which patients could benefit from this therapy. The evaluation of Beta-AR protein expression on tumour tissues might be a plausible strategy. In NSCLC, studies addressing the presence of these receptors are clearly lacking. In this retrospective clinicopathological study we aimed to evaluate the differences between resected tissue specimens from ADC and SCC in terms of the expression pattern of Beta1- and Beta2-AR in both, tumour and adjacent surrounding non-tumour tissues.

In addition, to understand the functional relevance of Beta-AR in NSCLC, we characterized Beta-AR in A549 cells, an established human NSCLC cell line, and evaluated the effects of adrenaline and of the selective Beta2-AR agonist isoprenaline on cell proliferation.

Materials and Methods

Patient Samples and Study Design

This retrospective study was based on the analysis of 80 patients with histologically confirmed diagnosis of primary NSCLC who received surgical treatment at the “Ospedale del Circolo”, Varese (Italy) from 1998 to 2015. The NSCLC cases were carefully selected in order to obtain the most homogeneous sample in terms of histologic subtype (40 ADC and 40 SCC) and clinical stage (10 of each clinical stage: from stage I to stage IV).

Patients with other simultaneous primary tumors or undergoing pre-operative chemotherapy and/or radiotherapy were excluded from the study. The following clinicopathological information were collected from hospital records: patient age, gender, tobacco/alcohol consumption, forced expiratory volume in 1 s (FEV1%), tumour size, differentiation grade, lymph node status, tumour location, clinical stage, and clinical follow-up (death from disease or from other causes). The TNM stage of each case was reclassified according to the 7th edition of the International Union Against Cancer

(UICC) criteria. The Research Ethics Committee from "Ospedale del Circolo", Varese (Italy) approved this study. Surgically resected specimens of NSCLC were obtained from the archives of Department of Pathology, and the formalin-fixed, paraffin-embedded tissue blocks were cut into 3 μm sections for haematoxylin and eosin (H&E) staining and immunohistochemistry analysis of Beta1 and Beta2-AR. The H&E slides of all the cases were reviewed by a pathologist with subspecialty training and experience in lung pathology (FF) (Bossard et al. 2011) who choose the more representative tumour tissue block for the immunohistochemical analysis.

Immunohistochemistry

Beta-AR expression was determined by immunohistochemical staining. Briefly, 3-μm sections of each paraffin-embedded tumour sample were mounted on poly-L-lysine coated slides, deparaffinised and hydrated through graded alcohols to water. After endogenous peroxidase activity inhibition, performed by dipping sections in 3% hydrogen peroxide for 20 min (min), sections were treated in 10 mM citrate buffer (pH 6.0) in a microwave for 10 or 20 min respectively depending of the antibody for Beta1-AR or Beta2-AR detection. For Beta1-AR detection the tissue sections were incubated for 1 h (h) at room temperature in a humidified chamber with the rabbit polyclonal anti-beta1 adrenergic antibody (#ab3442, Abcam, Inc., Cambridge, UK) diluted at 1:500. For Beta2-AR detection the tissue sections were incubated with the rabbit monoclonal anti-beta2 adrenergic receptor antibody (#ab182136, Abcam, Inc., Cambridge, UK) raised against a C-terminal peptide of the human Beta2-AR diluted at 1:500 overnight at 4 °C in a humidified chamber. Next, the sections were sequentially incubated with Primary antibody amplifier Quanto (UltraVision™ Quanto Detection System HRP DAB, Thermofisher) for 15 min, washed 3 times with TBS-Triton buffer PH 7.4 followed by incubation for 15 min with HRP Polymer Quanto from the same kit and washed with TBS buffer PH 7.4. The antigen-antibody reactions were revealed using 3,3′ diaminobenzidine tetra-hydrochloride (DAB, Sigma). Sections were counterstained with Harris haematoxylin before being dehydrated and mounted with a cover slip. Specificity controls consisted of substitution of the primary antibody with rabbit non-immune serum at the same dilution and use of control tissues with or without the appropriate antigen.

Immunohistochemical Scoring

Immunohistochemical sections were assessed using light microscopy, by two of the authors (M.Coe, A.M.C) who were blinded to the clinical characteristics and outcome of the patients. Positivity and intensity of Beta1- and Beta2-AR staining on NSCLC tissues were evaluated by semi-quantitative scoring using the H-score method for both tumour and non-tumour surrounding tissue (normal bronchial epithelium). The H-score is given by the application of the following algorithm:

$$\begin{aligned} \text{H}-Score = {} & (\%of\ cells \text{ at } 0) \times 0 \\ & + (\%of\ positive\ cells \text{ at } intensity\ 1+) \times 1 \\ & + (\%of\ positive\ cells \text{ at } intensity\ 2+) \times 2 \\ & + (\%of\ positive\ cells \text{ at } intensity\ 3+) \times 3. \end{aligned}$$

The ordinal values correspond to the intensity level (0 = none, 1 = weak, 2 = moderate, and 3 = strong).

With four intensity levels, the resulting score ranges from 0 (no staining in the tumour) to 300 (diffuse intense staining of the tumour).

Cell Culture

The human NSCLC A549 cell line was obtained from the European Collection of Cell Cultures (ECACC, Porton Down, UK). Cells were routinely cultured in RPMI 1640 (EuroClone, Milan, Italy) supplemented with 10% FBS (EuroClone, Milan, Italy) and 100 U/ml penicillin/streptomycin (EuroClone) at 37 °C in a moist atmosphere of 95% O_2/5% CO_2. Cells were cultured until sub-confluent cultures were reached and then split 1:10 using trypsin/EDTA and plated in 75 cm^2 flasks.

Expression of mRNA for Beta-AR by Real-Time PCR

Cells were cultured during 7 days and the total RNA of the cells on days 3, 5 and 7 after seeding was extracted. Real-time PCR of Beta-AR mRNA was performed according to a previously reported method with modifications Scanzano et al. (2015). Briefly, cells were resuspended in Perfect Pure RNA lysis buffer and total RNA was extracted using PerfectPure RNA Cell KitTM. Total mRNA obtained was reverse-transcribed using iScriptTM Reverse Transcription Supermix and the cDNA was then amplified using SsoAdvancedTM Universal Probes Supermix for the analysis of ADRBQ, ADRB2 and ADRB3 gene expression, and with SsoAdvancedTM Universal SYBR® Green Supermix for analysis of TH gene expression.

Flow Cytometric Analysis of Cell Proliferation

Cell proliferation was determined by staining DNA with propidium iodide (PI, Sigma) according to previously described methods with modifications, (Cosentino et al. 2007; Spagnuolo et al. 2014). To this end, A549 cells were

harvested, washed with PBS by centrifugation at 600 g for 5 min, and fixed / permeabilized with 1.5 ml of 70% ice-cold ethanol for 3.5 h at 4 °C. After washing, pellets were resuspended in 50 μl of ribonuclease A solution (RNAse, Sigma) at the final concentration of 100 μg/ml and incubated at 37 °C for 30 min. Cell suspensions were then added 0.6 ml PBS containing 50 μg/ml PI and incubated at room temperature in the dark for 45 min. Flow cytometric analysis of DNA content was carried out using a BD FACSCanto II flow cytometer (Becton Dickinson Italy, Milan, Italy) with FACSDiva software (version 6.1.3). Fluorescence signal of PI (FL3) was collected on a linear scale using a 670-LP filter. At least 25,000 events/sample were acquired and gated on a standard bi-parametric dot plot (doublet discriminator plot) FL3-A vs FL3-H to distinguish single cells from doublets or aggregates. Each DNA histogram was analysed with FlowJo software (version 8.3.2) that fits cell cycle data using mathematical models to define the percentages (%) of cells in G0/G1, S and G2/M phases of the cell cycle. For the purpose of the present study, the percentage of cells in the S phase of the cell cycle, i.e. the part of the cell cycle in which DNA is replicated, was taken as an index of cell proliferation.

Treatments

The (−)adrenaline (Sigma-Aldrich) and the selective Beta2-AR agonist (−)-isoproterenol hydrochloride (Sigma-Aldrich) were prepared in 10^{-2} M stock solutions into culture serum-free medium. In each experiment, an appropriate negative control was included.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics version 24 for mackintosh (IBM corp., NY, USA). Chi-squared or Fisher's exact tests were used to examine the association between the categorical variables. H-scores for Beta1- and Beta2-AR were analysed as continuous variables. The normality of distributions of all continuous variables were analysed by the Kolmogorov–Smirnov test. Homogeneities of data were controlled by Levene's test. The normality and the homogeneity tests were used to decide which statistical methods to apply in comparing groups. Differences between H-scores were evaluated by Independent Samples Student's t test or Mann-Whitney rank sum test, as appropriate. The comparisons between tumour and matched surrounding non-tumour tissues was determined by using Wilcoxon matched-pairs signed rank test. The overall survival (OS) was defined as the time from tumour resection to death from any cause. The univariate analysis for all possible prognostic factors was determined by using the Cox proportional hazards regression model. Survival analyses were assessed using Kaplan–Meier plots and log-rank tests. GraphPad Prism 6 (San Diego, CA) was used for graphical representations.

Results

Patients Characteristics

Eighty patients who received surgical treatment for primary NSCLC at the Ospedale di Circolo di Varese (Italy) were included in this study. One of the resected tissue specimens was not suitable for analysis and therefore, only 79 samples were considered for Beta1- and Beta2-AR protein expression by IHC. Table 1 presents the clinical characteristics organized by the histologic subtypes. There were several differences in baseline characteristics between ADC and SCC patients. In particular, we have shown that there were higher proportion of SCC patients with age ≥ 65 years (72.5% vs 48.7; $P = 0.030$), higher frequency of men (95% vs 74.4%; $P = 0.011$) and were more likely to be smokers or former smokers (65.0% vs 46.2% or 35.0% vs 28.2%; $P = 0.003$).

Immunohistochemical Analysis

Beta-AR Staining

All matched surrounding non-tumour tissues (bronchial epithelium) had positive cell staining for both Beta1- and Beta2-AR which served as an internal positive control. For both receptors, the immunoreactivity was prevalently observed in the apical cytoplasm (see NT in Figs. 1 and 2) and the intensity varied from weak to moderate for Beta1-AR and prevalently moderate for Beta2-AR. On tumour cells, the immunoreactivity for both receptors was observed in the cytoplasm and along the membrane. In most cases, the Beta1-AR staining was diffuse sometimes granular and varied in intensity from weak to moderate (Fig. 3a, c) whereas for Beta2-AR the staining was also granular, but the intensity varied from moderate to strong (Fig. 4b, c).

To compare Beta1- and Beta2-AR protein expression between tumour and matched surrounding non-tumour tissue, the IHC was performed in 69 paired NSCLC tissues, from those 32 were SCC and 37 were ADC. The H-scores were calculated for each tissue, as previously described in the method section.

Beta-AR Staining in ADC and in their Matched Surrounding Non-tumour Tissues

Beta1- and Beta2-AR are differently expressed in ADC. A lower Beta1-AR expression was observed in 84% (31/37) of the ADC tissues (mean ± SD = 82.1 ± 60.8) when compared with the matched surrounding non-tumour tissues (145.9 ± 55.8; $P < 0.0001$; Fig. 1a, b). In contrast, a higher expression

Table 1 Patients and disease characteristics

Characteristics		Total N (%) 79 (100)	Histologic subtype SCC N (%) 40 (50.6)	Histologic subtype ADC N (%) 39 (49.4)	P value
Age	< 65	31 (39.2)	11 (27.5)	20 (51.3)	**0.030**
	≥ 65	48 (60.8)	29 (72.5)	19 (48.7)	
Gender	Male	67 (84.8)	38 (95.0)	29 (74.4)	**0.011**
	Female	12 (15.2)	2 (5.0)	10 (25.6)	
Clinical stage [a)]	[IA – IB]	22 (28.2)	12 (30.8)	10 (25.6)	0.733
	[IIA - IIB]	19 (24.4)	10 (25.6)	9 (23.2)	
	[IIIA - IIIB]	21 (26.9)	11 (28.2)	10 (25.6)	
	[IV]	16 (20.5)	6 (15.4)	10 (25.6)	
Differentiation Grade	G1 - well	0 (0.0)	0 (0.0)	0 (0.0)	0.292
	G2 – moderate	55 (69.6)	30 (75.0)	25 (64.1)	
	G3 - poorly	24 (30.4)	10 (25.0)	14 (35.9)	
FEV1% [b)]	< 80	26 (33.8)	16 (41.0)	10 (25.6)	0.172
	≥ 80	51 (66.2)	23 (59.0)	28 (71.8)	
BMI*	< 25	30 (45.5)	13 (41.9)	17 (48.6)	0.587
	≥ 25	36 (54.5)	18 (58.1)	18 (51.4)	
Smoking history	Yes	44 (55.7)	26 (65.0)	18 (46.2)	**0.003**
	Former	25 (31.6)	14 (35.0)	11 (28.2)	
	No	10 (12.7)	0 (0.0)	10 (25.6)	

SCC, squamous cell carcinoma; ADC, adenocarcinoma; FEV1%, forced expiratory volume in 1 s; BMI, Body mass index; Bold values denote statistical significance at $p < 0.05$ level

[a)] 1 SCC case with non-identifiable clinical stage (T3 Nx)

[b)] Excluding patients with lost records

of Beta2-AR was detected in 76% (28/37) of ADC tissues (200.1 ± 63.40) in comparison with matched surrounding non-tumour tissues (167.6 ± 47.5; $P = 0.0034$; Fig. 1c, d). Complete absence of staining for Beta1-AR was observed in

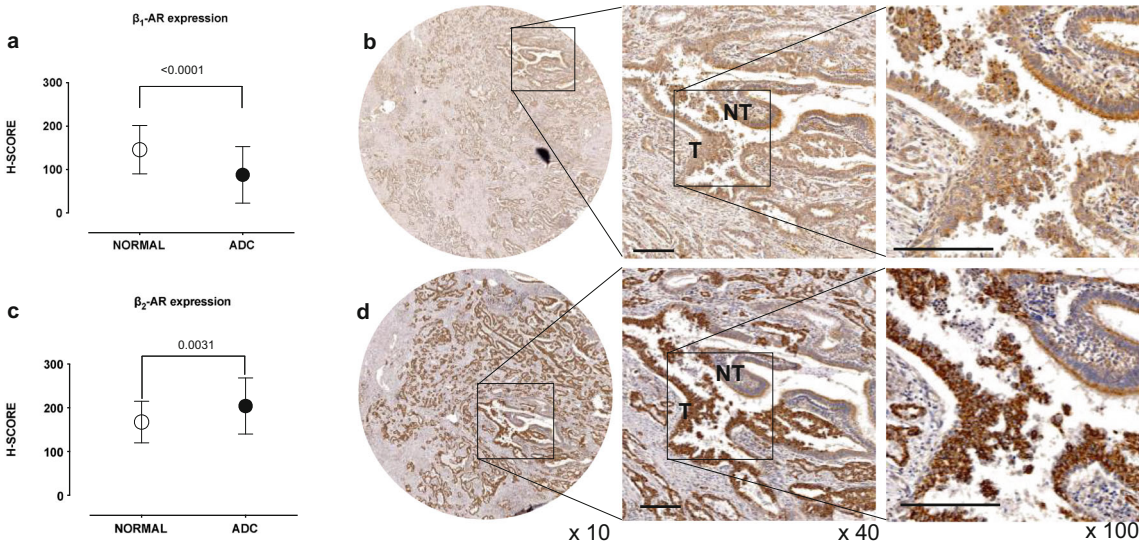

Fig. 1 Expression of Beta1 and Beta2-AR in human ADC and in their matched surrounding non-tumour tissues. a. and **c.** Box–plots representing the H-score differences between NT (non-tumour) and T (tumour) in ADC (pairs $n = 37$) respectively for Beta1 and Beta2-AR. Each set of box and whiskers represents the median, first and third quartiles, and the maximum and minimum values. **b.** and **d.** representative Immunohistochemical staining illustrating the expression of Beta1-AR and Beta2-AR, respectively. Statistical analysis was performed by the Wilcoxon matched-pairs signed rank test and the resulting P value is indicated in the graph . Magnifications are respectively: 10x, 40x and 100x. Scale bars correspond to 200 μm. T = tumor tissue; NT = non-tumor tissue

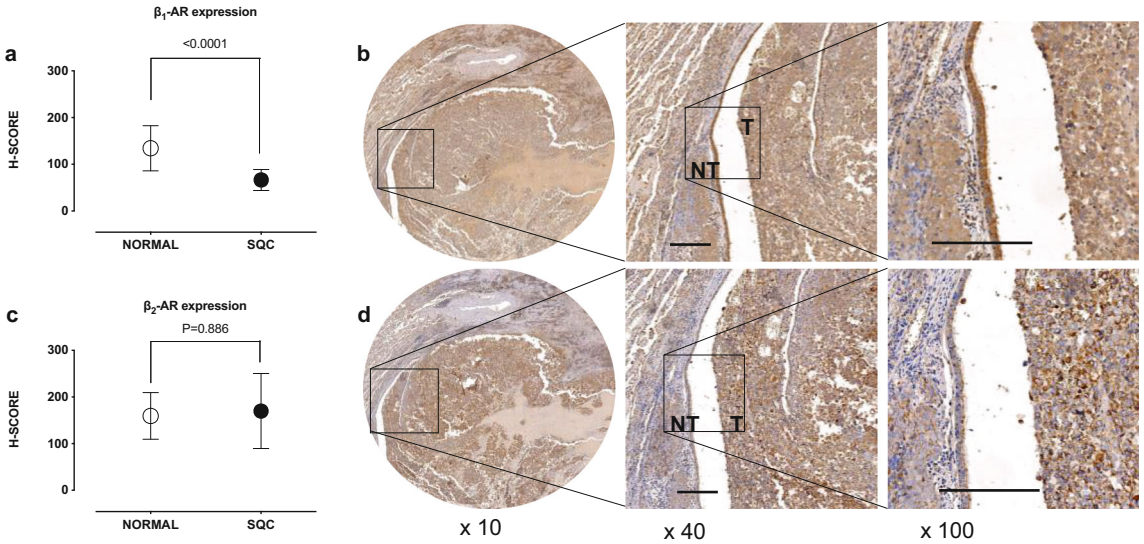

Fig. 2 Expression of Beta1 and Beta2-AR in human SCC and in their matched surrounding non-tumour tissues. a. and **c.** Box-plots representing the H-score differences between NT (non-tumour) and T (tumour) in SCC (pairs $n = 32$) respectively for Beta1 and Beta2-AR. Each set of data are presented as the mean ± SD of expression measured in normal (empty circle) and tumour (filled circle) tissues. **b.** and **d.** representative Immunohistochemical staining illustrating the expression of Beta1-AR and Beta2-AR, respectively. Statistical analysis was performed by the Wilcoxon matched-pairs signed rank test and the resulting P value is indicated in the graph. Magnifications are respectively: 10x, 40x and 100x. Scale bars correspond to 200 μm. T = tumor tissue; NT = non-tumor tissue

7.7% (3/39) ADC tumours while for Beta2-AR the positivity was observed in 100% (39/39) of the ADC tumours.

Beta-AR Staining in SCC and in their Matched Surrounding Non-Tumour Tissues

For Beta1-AR expression, the same trend was observed in SCC, being once again lower expressed in 81% (26/32) of the tumour tissues (68.7 ± 70.2) when compared with matched surrounding non-tumour tissue (134.4 ± 48.3; $P < 0.001$); Fig. 2a, b). For Beta2-AR, no significant difference was observed between the tissues (SCC: 159.4 ± 49.9 vs NT: 161.4 ± 78.6; Fig. 2c, d). Complete absence of staining for Beta1-AR was observed in 15% (6/40) SCC tumours whereas Beta2-AR was once again positive in 100% (40/40) of the SCC cases.

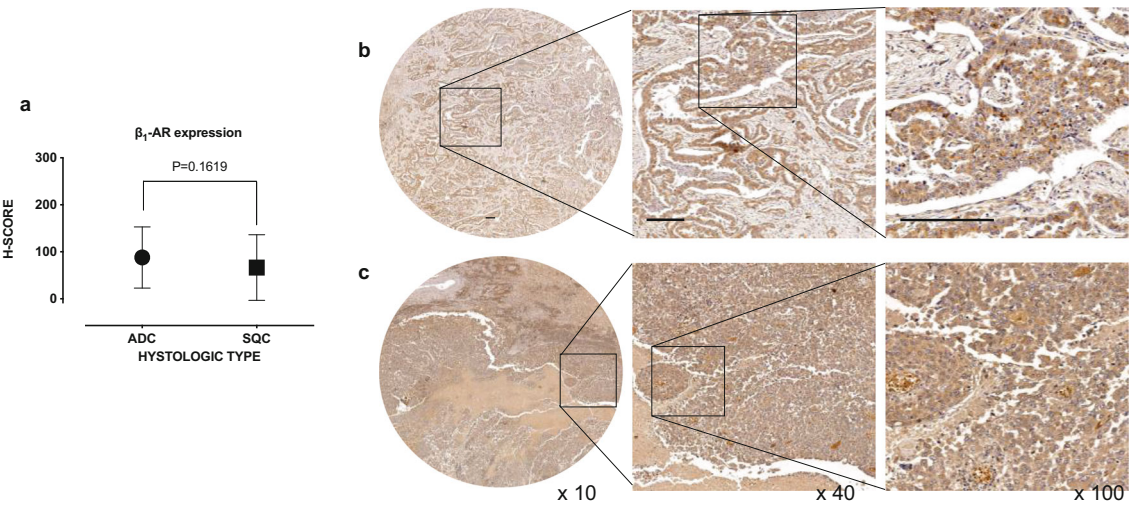

Fig. 3 Expression of Beta1-AR in ADC and SCC Panel a: Box–plot for H-scores. Each set of box and whiskers represents the median, first and third quartiles, and the maximum and minimum values. Panels **b** and **c**: representative Immunohistochemical staining illustrating the expression of Beta2-AR in ADC ($n = 39$) and SCC ($n = 40$) respectively. Data are presented as the mean ± SD of expression measured in ADC (circle) and SQC (square) tumours. Statistical analysis was performed using Student's *t* test for unpaired data, and the resulting P value is indicated in the graph . Magnifications are respectively: 10x, 40x and 100x. Scale bars correspond to 200 μm. T = tumor tissue; NT = non-tumor tissue

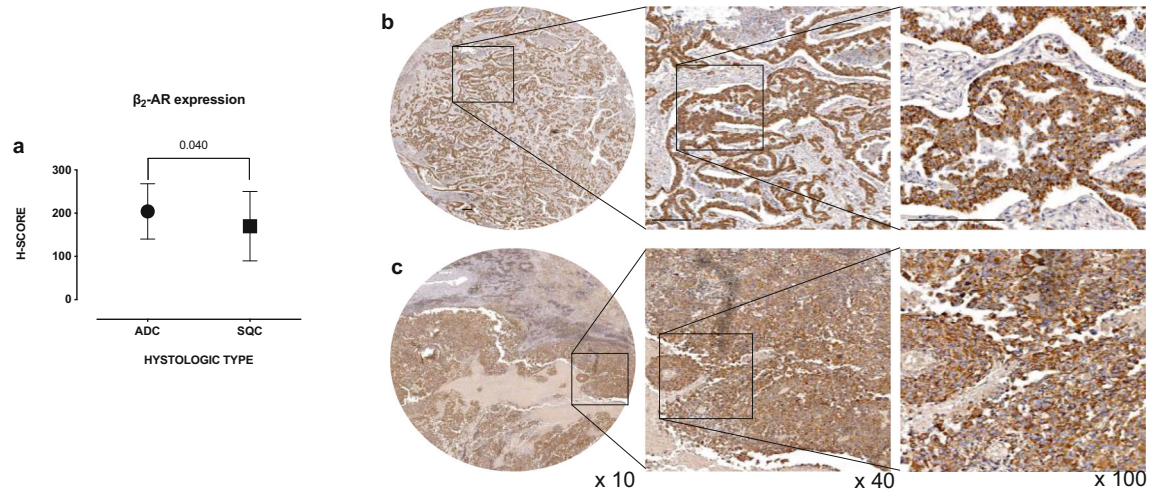

Fig. 4 Expression of Beta2-AR in ADC and SCC Panel a: Box–plot for H-scores. Each set of box and whiskers represents the median, first and third quartiles, and the maximum and minimum values. Panels **b** and **c**: representative Immunohistochemical staining illustrating the expression of Beta2-AR in ADC (n = 39) and SCC (n = 40) respectively. Data are presented as the mean ± SD of expression measured in ADC (circle) and SQC (square) tumours. Statistical analysis was performed using Student's *t* test for unpaired data, and the resulting P value is indicated in the graph . Magnifications are respectively: 10x, 40x and 100x. Scale bars correspond to 200 μm. T = tumor tissue; NT = non-tumor tissue

Comparison of Beta-AR Staining in ADC vs SCC

As shown in Fig. 3, Beta1-AR is expressed at low levels in both histologic subtypes. In ADC (Fig. 3b) the H-score obtained for Beta1-AR was 87.9 ± 65.2 ($N = 39$) and in SCC (Fig. 3c) was 66.4 ± 69.7 ($N = 40$). Conversely, Beta2-AR is highly expressed in both histologic subtypes (Fig. 4a) being clearly overexpressed in ADC (Fig. 4b) (204.0 ± 64.0; N = 39) when compared with SCC (Fig. 4c) (169.9 ± 80.3; N = 40) ($P = 0.040$).

Tumour-Related Characteristics According to Beta1 and Beta2-AR Expression

To determine the clinical significance of Beta1- and Beta2-AR expression in NSCLC, the H-scores obtained in tumour tissues were compared between the dichotomous clinicopathological parameters for each histologic subtype (Table 2). Among SCC patients Beta1-AR was not associated with any variable. Conversely, in ADC patients Beta1-AR expression was higher in older patients (age ≥ 65 years) ($P = 0.004$), in females ($P = 0.032$) and in samples from subjects with lymph node involvement ($P = 0.050$). Regarding Beta2-AR, no statistical significant differences were detected between expression levels and the clinicopathological features, neither in ADC nor in SCC.

Outcome According to the Beta1 and Beta2-AR Expression

The day of surgery was considered the starting day for measuring the postoperative survival. Median follow-up time was 825.5 days (range: 33–5053 days) for SCC patients and 1444.5 (range: 81–5596 days) for ADC patients. The overall 5-year survival rate for SCC patients was 45.0% and 51.4% for ADC patients. Regarding the cancer specific 5-year survival rates was 56.8% for SCC and 62.2% for ADC (data not shown).

To further evaluate the possible prognostic value of Beta1- and Beta2-AR expression in ADC and SCC, we explored the association between their protein expression levels and the overall 5-year survival rates by Kaplan-Meier analysis (log-rank test) and Cox proportional hazards regression model (S1, S2 and S3 Tables). The cut-off values to allocate patients to high and low expression levels were determined based on the median values of the H-scores obtained for each receptor in each histologic subtype, as indicated in S2 and S3 Tables. It was observed that neither Beta1- nor Beta2-AR was associated with survival for none of the histologic subtypes. Furthermore, none of the variables were significantly associated with overall survival (OS) in our SCC patient's cohort. Conversely, as expected, for the ADC cohort it was observed that clinical stage (HR: 2.985, 95% CI: 1.144–7.785; $P = 0.025$) and the presence of metastasis (HR: 7.892; 95% CI: 2.718–22.833 $P < 0.0001$) were significantly associated with poor OS.

Expression of ADRB in the A549 Cell Line

The present experiments were undertaken to confirm the expression of ADRB1, ADRB2, and ADRB3 during the period of culture in basal conditions. As shown in Fig. 4, real-time PCR revealed that A549 cells expresses different levels of ADRB. Curiously enough, it was observed that whereas ADRB1 decreased its expression from day 3 to day 7, ADRB2 maintained unchanged its high levels of expression. ADRB3 was not detectable.

Table 2 Tumour-related characteristics according to Beta1 and Beta2-AR expression in squamous-cell carcinoma (SCC) and adenocarcinoma (ADC)

		Beta1-AR				Beta2-AR			
		SCC		ADC		SCC		ADC	
		H-SCORE Median (IQR)	p value	H-SCORE Median (IQR)	p value	H-SCORE Median (IQR)	p value	H-SCORE Median (IQR)	P value
Age	<65 ≥65	60 (28–149) 30 (3–120)	0.309	60 (9–100) 113 (60–156)	**0.004**	158 (99–270) 170 (100–235)	0.978	225 (200–265) 180 (118–250)	**0.010**
Gender	Male Female	50 (10–120) 100 (0.0–200)	0.926	70 (10–110) 115 (68–176)	**0.032**	170 (100–235) 198 (95–300)	0.664	220 (170–265) 200 (144–231)	0.594
BMI	<25 ≥25	30 (8–155) 70 (10–105)	0.748	75 (53–118) 93 (65–143)	0.346	110 (65–248) 160 (100–205)	0.689	180 (154–210) 225 (158–270)	0.302
FEV %	<80 ≥80	30 (8–105) 70 (10–150)	0.650	60 (30–99) 100 (65–145)	0.232	100 (70–203) 180 (103–245)	0.141	200 (95–268) 205 (168–245)	0.982
Tumour Location	Peripheral Central	30 (2–85) 50 (10–150)	0.086	70 (27–115) 109 (73–139)	0.483	165 (101–268) 175 (100–235)	0.897	220 (168–250) 205 (153–278)	0.859
Clinical stage	[IA - IB] or [IIA -IIB] [IIIA - IIIB] or [IV]	50 (10–115) 50 (3–150)	0.909	110 (60–150) 70 (33–93)	0.221	200 (98–275) 145 (101–195)	0.222	190 (150–265) 223 (170–250)	0.656
pT	T1 - T2 T3 - T4	50 (10–125) 50 (1–150)	0.613	90 (48–136) 50 (25–55)	0.167	190 (100–270) 135 (95–145)	0.226	210 (161–250) 250 (220–275)	0.235
pN	N0 N1-N2	30 (8–118) 50 (5–115)	0.338	128 (83–180) 60 (13–102)	**0.050**	148 (91–273) 170 (100–230)	0.891	188 (118–269) 220 (170–250)	0.642
Metastasis	M0 M1	50 (8–130) 10 (0–75)	0.191	90 (60–128) 50 (10–103)	0.508	170 (100–260) 165 (126–185)	0.515	205 (157–245) 250 (170–273)	0.294
Tumour Dimension (cm)	<3 ≥3	50 (5–110) 40 (5–143)	0.754	60 (11–99) 102 (60–135)	0.210	200 (135–265) 145 (91–231)	0.200	215 (166–261) 210 (170–250)	0.904
Differentiation Grade	G2 – moderate G3 – poorly	50 (10–130) 60 (0.3–138)	0.415	90 (13–115) 85 (48–143)	0.378	175 (100–243) 123 (96–254)	0.580	225 (170–265) 190 (143–250)	0.186

H-scores were used to compare the immunohistochemical staining of protein expression levels. Data are presented as Median (IQR, interquartile range from 25th and 75th percentiles) for H-score staining intensities. Bold values denote statistical significance at $p < 0.05$ level

Effects of Adrenaline and Isoprenaline on A549 Cell Proliferation

A549 cells were treated with an increasing range of concentrations (10^{-7} to 10^{-4} M) of adrenaline and the selective Beta2-AR isoprenaline to obtain complete concentration-response relationships for each drug. As shown in Fig. 5, both adrenaline and isoprenaline increased the percentage of A549 cells in the S phase of the cell cycle in a concentration-dependent manner. The EC_{50} (i.e. the effective concentration that increased proliferation to 50% of the maximal effect of each agonist) was 1.1×10^{-5} M (CI 95%, 3.0×10^{-6} M - 3.8×10^{-5} M) for adrenaline, and 2.2×10^{-6} M (CI 95%, $3.8\ 10^{-7}$ M to 1.2×10^{-5} M) for isoprenaline.

Discussion

NSCLC represents the most common type of lung cancer, accounting for approximately 80% of the cases among which ADC and SCC are the two most prevalent histological subtypes (Novello et al. 2016). Besides the advanced progresses in experimental and clinical oncology have been made, the prognosis of both histologic subtypes remains very poor. Therefore, to better understand its progression and better predict the clinical outcome would be extremely useful the identification of novel targets associated with NSCLC; this can possibly help to find successful and innovative therapeutic approaches which at present are clearly lacking for this tumour. The presence and function of Beta-AR in human lung is crucial for the normal physiologic functions namely bronchodilation and smooth muscle relaxation (Bossard et al. 2011; Daly and Mcgrath 2011), however their presence on human lung tumours and their clinicopathological significance remain unclear.

Given the ability of Beta-AR signalling in modulating tumour microenvironment by multiple mechanisms and possibly influencing on the major hallmarks of cancer, Beta-AR antagonism by Beta-blockers, has been indicated as an opportunity for drug repurposing as a novel class of anti-tumour agents (Ji et al. 2012; Nagaraja et al. 2013; Pantziarka et al. 2016). Indeed, the functional relevance of this signalling as possible new target can help to find new therapeutic approaches for this aggressive tumor.

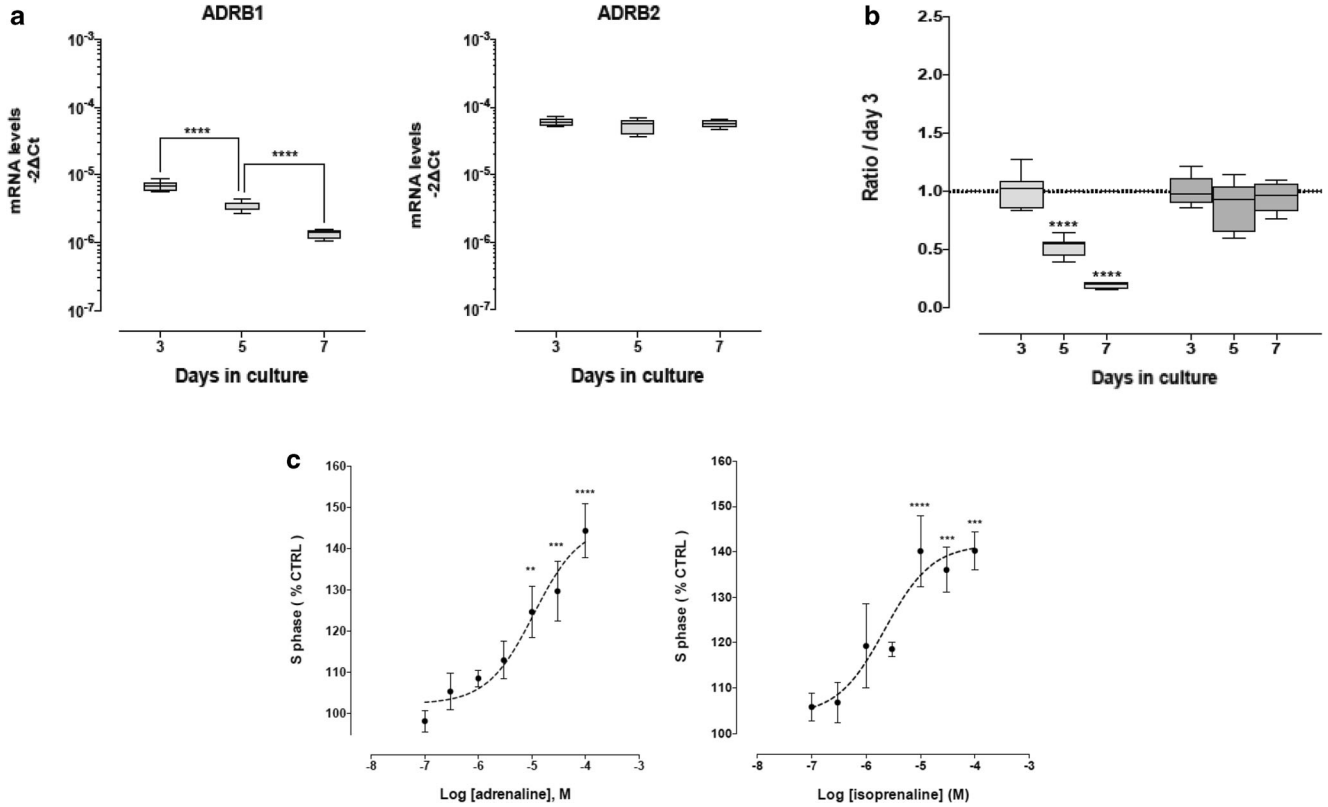

Fig. 5 ADRB mRNA expression in A549 cell line during the growth curve, on day 3, 5 and 7. Panel a: Data are expressed as 2-ΔΔCt and boxes indicate median with 25th–75th percentiles and whiskers indicate minimum and maximum values from 3 separate experiments. Differences in ADBR mRNA expression levels during the growth of the culture were analysed by one-way ANOVA followed by Tukey's post-test. **** = $P < 0.0001$. **Panel b:** Data are expressed as ratio of day 3 and boxes indicate median with 25th–75th and whiskers indicate minimum and maximum values from 3 separate experiments. Differences on ADBR mRNA expression levels between day 5 and 7 with day 3 were analysed by one-way ANOVA followed by Tukey's post-test. **** = $P < 0.0001$. **Panel c:** Concentration- response curves for the effect of isoprenaline and adrenaline on the proliferation of cultured A549 cells. Proliferation was measured as the fraction of cells in S phase of the cell cycle and expressed as % of control. Each point represents the mean ± S.E.M. of at least 5 independent experiments. Curves represent the best fit of the sigmoidal concentration–response curves calculated by non-linear regression analysis. Differences were analyzed by one-way ANOVA followed by Turkey's post-test. ** = $P < 0.01$; *** = $P < 0.0001$; **** = $P < 0.00001$

Despite certain reports have been providing several insights regarding the function of β-AR in lung cancer progression (Schuller and Cole 1989; Schuller et al. 1999; Schuller and Cekanova 2005; Laag et al. 2006; Al-wadei et al. 2012; Hu et al. 2016), the global picture is yet not fully understood. By using one of the most well known and widely used model of NSCLC, A549 cells, which are ADC-derived, we aimed to collect information on the expression and the functional relevance of Beta-AR in these cells. Our results show that these cells express both ADRB1 and ADRB2 mRNA at different levels whereas ADRB3 presence was not detected. Results are in line with the recent data reported by Ping Hu et al. (2016), (Hu et al. 2016) showing that ADRB2 is the prominent Beta-AR subtype on these cells. Interestingly, the same trend was observed in our clinicopathologic study when evaluating ADC tissues. Likewise,we found that Beta1-AR was expressed at low levels and Beta2-AR was highly expressed. Moreover, our results show that both AD and ISO could significantly enhance the proliferation of the A549 cells in a concentration-dependent manner. This finding is consistent with Ping Hu et al. (2016), (Hu et al. 2016) that suggest that the increase of cell proliferation promoted by ISO is mediated by Beta2-AR.

Beta-blockers have shown to be effective in the control of tumour progression with pleiotropic effects on the primary tumour, its surrounding microenvironment and metastatic target sites (Quốc Lu'o'ng and Nguyễn 2012). This therapeutic approach is even more appealing given their well-known and well characterized safety profile, inexpensive cost and widespread use in clinical setting. Hence, having knowledge about the Beta-AR expression status and its pattern of expression in the tumours could help us selecting the patients with potential to benefit from Beta-blocker therapy and better outline the Beta-blocker class to use. In addition, it has been demonstrated that there are significant differences in the underlying mechanisms between ADC and SCC progression, which encouraged us to consider that might exist relevant differences in Beta-AR expression between them (Raponi et al. 2006; Faruki

et al. 2017). To address this, we carried out a retrospective clinicopathological study to evaluate the Beta1- and Beta2-AR expression in both ADC and SCC tissues comparing them with their matched surrounding non-tumours tissues to better describe their presence on NSCLC.

Our results provide strong evidences for the differential expression of Beta1- and Beta2-AR across the histological subtypes of NSCLC under study. We found that Beta1-AR expression is present at low levels in both SCC and ADC. Similarly, when compared with the matched surrounding non-tumour tissues, Beta1-AR expression level was significantly lower in both histologic subtypes. On the contrary, Beta2-AR is highly expressed in both histologic subtypes but clearly highly expressed in ADC when compared with SCC. Likewise, we found that Beta2-AR expression is significantly higher in ADC when compared with matched surrounding non-tumour tissue but the same was not observed in SCC. These results are consistent with previous studies showing that Beta2-AR are actually highly expressed in ADC from NSCLC (Yazawa et al. 2016a; Rains et al. 2017).

It was also noted that both receptors were expressed in most tumour-associated stromal cells, particularly in macrophages which suggests that catecholamines could affect tumour microenvironment by different ways as described by some authors (Eng et al. 2014; Cole et al. 2015).

During the last decade, several studies have been published reporting the Beta-AR expression in a wide range of human tumours (Ramberg et al. 2008; Bravo-Calderón et al. 2012; Chen et al. 2012; Liu et al. 2015; Takahashi et al. 2016; Yazawa et al., 2016a, b; Rains et al. 2017). The techniques and scoring methods used to assess Beta-AR expression levels have varied among studies, with most using qRT-PCR, Western blotting, and IHC. In this study, we choose the IHC and H-score scoring method to have the global picture of the staining in terms of localization as well as positivity and intensity, providing a continuous variable ranging from 0 to 300. Considering that no recognized IHC technique and scoring method has yet been generally accepted, the expression levels of Beta-AR reported so far, may vary depending on the methodology used. Over the last years, with the advances in proteomics and transcriptomics in oncology, it is sufficiently well described that there are discrepancies in terms of mRNA levels and corresponding protein levels (Vogel and Marcotte 2013). In fact, it has been estimated that only about 30% - 60% of variations in the protein levels can actually be explained by the corresponding changes in mRNA levels (Vogel and Marcotte 2013). This knowledge reinforces even more the importance of directly assessing the protein levels in the search for new biomarkers or drug targets. Although the evaluation of Beta-AR protein expression does not predict the absolute responsiveness to Beta-blocker treatments, it could be extremely helpful in the identification of negative tumours which probably will not benefit from the treatment and in the selection of those presenting positivity and that theoretically could be more sensible to the Beta-blocker therapy. For instance, Beta-blockers have shown to be clinically effective in the vascular tumours such as infantile haemangioma, which are well characterized tumours highly expressing Beta-AR (Chisholm et al. 2012). In fact, since 2014 Propranolol, a non-selective Beta-blocker, has been repurposed and FDA-approved as the first-line therapy for the treatment of this disease (Lou et al. 2013).

In line with these latter evidences, and based on our results, it is conceivable to suggest that among NSCLC, ADC may have the greater benefit from non-selective Beta-blockers.

Equally important, the high expression of Beta-AR reported in multiple malignancies (Rains et al. 2017) it is also being associated with and poorer clinicopathological features and consequently poorer prognosis (Ramberg et al. 2008; Bravo-Calderón et al. 2012; Chen et al. 2012; Liu et al. 2015; Takahashi et al. 2016; Yazawa et al. 2016b). For instance, during the last few years Shimizu and *colleagues* have been reported that Beta2-AR expression is significantly associated with several poor clinicopathological features namely, tumour size, differentiation, lymphatic permeation, and vascular invasion and having been identified as an independent prognostic factor for in melanoma (Yazawa et al. 2016b), gastric (Takahashi et al. 2016) and NSCLC (Yazawa et al. 2016a). Conversely, in the present study, we did not find any particular and significant association between Beta-AR expression with the clinicopathological parameters. However, it was possible to observe, although not statistically significant, there is a trend towards higher Beta2-AR expression level in advanced stages of ADC (clinical stage III and IV, T factor 3–4, lymphatic node involvement, and metastasis). Furthermore, in none of the post-hoc survival analysis performed, the results support Beta2-AR overexpression as an independent prognostic factor. However, it is very likely that the absence of statistical significance in the survival analysis had been due to the scoring method of Beta-AR expression and to the stratification of the variables (by histologic subtype, clinical stage, H-score high/low) which substantially affected the sample sizes among the groups.

There are several limitations that should taken into account in the interpretation of the results reported in this clinicopathological study. First, the fact that it is a single centre retrospective cohort may have added some bias to the results; Second, given the absence of other studies reporting Beta-AR expression using H-score method, makes difficult to determine an optimal cut-off point for establishing what should be considered a high or low expression for these receptors. Third, the careful selection of the patient's tissues included in this study, simultaneously in terms of histologic subtype and clinical stage limited the sample size.

Overall, this clinicopathological study highlights the differential expression of Beta1- and Beta2-AR among ADC and

SCC in NSCLC. Pharmacological inhibition using non-selective Beta-blockers by repurposing them in oncologic setting might be a suitable therapeutic strategy for ADC. In the light of these evidences, further studies are warranted to clarify if Beta-blockers therapeutic effects are directly associated with Beta-AR expression status.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

Ethics Approval and Consent to Participate All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional research committee. Permission was obtained from the local ethics committee (Ospedale del Circolo - Varese) for this retrospective analysis. For this type of study formal consent is not required. All data were recorded and analysed anonymously.

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