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Research paper

The components of somatostatin and ghrelin systems are altered in neuroendocrine lung carcinoids and associated to clinical-histological features

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ABSTRACT

Background: Lung carcinoids (LCs) are rare tumors that comprise 1–5% of lung malignancies but represent 20–30% of neuroendocrine tumors. Their incidence is progressively increasing and a better characterization of these tumors is required. Alterations in somatostatin (SST)/cortistatin (CORT) and ghrelin systems have been associated to development/progression of various endocrine-related cancers, wherein they may become useful diagnostic, prognostic and therapeutic biomarkers.

Objectives: We aimed to evaluate the expression levels of ghrelin and SST/CORT system components in LCs, as well as to explore their putative relationship with histological/clinical characteristics.

Patients and methods: An observational retrospective study was performed; 75 LC patients with clinical/histological characteristics were included. Samples from 46 patients were processed to isolate mRNA from tumor and adjacent non-tumor region, and the expression levels of SST/CORT and ghrelin systems components, determined by quantitative-PCR, were compared to those of 7 normal lung tissues.

Results: Patient cohort was characterized by mean age 53 ± 15 years, 48% males, 34% with tobacco exposure; 71.4/28.6% typical/atypical carcinoids, 21.7% incidental tumors, 4.3% functioning tumors, 17.7% with metastasis. SST/CORT and ghrelin system components were expressed at variable levels in a high proportion of tumors, as well as in adjacent non-tumor tissues, while a lower proportion of normal lung samples also expressed these molecules. A gradation was observed from normal non-neoplastic lung tissues, non-tumor adjacent tissue and LCs, being SST, sst4, sst5, GHS-R1a and GHS-R1b overexpressed in tumor tissue compared to normal tissue. Importantly, several SST/CORT and ghrelin system components displayed significant correlations with relevant clinical parameters, such as necrosis, peritumoral and vascular invasion, or metastasis.

Conclusion: Altogether, these data reveal a prominent, widespread expression of key SST/CORT/ghrelin system components in LCs, where they display clinical-histological correlations, which could provide novel, valuable markers for NET patient management.

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1. Introduction

Lung neuroendocrine tumors (LNETs) represent 20–30% of all neuroendocrine tumors [1–3]. The 2015 World Health Organization (WHO) classifies lung neuroendocrine tumors in low-grade typical carcinoid (TC), intermediate-grade atypical carcinoid (AC) and high-grade large cell neuroendocrine carcinomas (LCNEC) and small cell carcinoma (SCLC) [4]. Although these neoplasms share morphological, immunohistochemical and ultrastructural features, there are significant clinical, prognostic and therapeutic differences between subtypes. Indeed, high-grade tumors are very aggressive and display poor prognosis, while lung carcinoids (LCs, including TCs and ACs) have been less characterized, with a less standardized clinical management, apart from surgical resection or chemoradiotherapy [5,6].

LCs display an incidence of 5–10 cases per million population/year [7,8], and 5–15% of multiple neuroendocrine neoplasia 1 (MEN1) patients [9,10]. Compared to other NETs, a lower proportion of LCs exhibits hormone hypersecretion, such as vasoactive intestinal peptide, adrenocorticotropic hormone (ACTH), and diuretic hormone, wherein the most common hormonal syndrome is the ectopic ACTH syndrome [11]. Paraneoplastic syndromes, including inappropriate antidiuretic hormone secretion, are even less frequent [9]. Importantly, LCs prognosis is tightly correlated to histotype, as TCs have a 5-year survival rate of 87%, presenting regional lymph node metastasis in 10–15% and distant metastases in 3–5% of cases. In contrast, ACs are more aggressive, with frequent nodal and distant metastases (20–50% respectively) and a 5-year survival of 60% [3,12,13]. Anyway, the only curative treatment for LCs is radical surgery [14].

The heterogeneity of these neoplasms, their different clinical behavior, and the possibility of recurrence or long-term metastasis, emphasize the importance of identifying new diagnostic and therapeutic markers, which could improve the diagnosis, prognosis and/or treatment of these patients. Accordingly, alterations in the regulatory neuroendocrine systems comprised by somatostatin (SST)/cortistatin (CORT), ghrelin and their receptors (ssts and GHSRs, respectively) have been associated to the development/progression of various endocrine-related cancers. However, their expression has not been systematically characterized in LCs.

SST and CORT are two highly related neuropeptides that exert a plethora of physiological, often inhibitory, functions, by acting through their so-called SST receptors (sst1-5) [15–18], which regulate, among other activities, cell proliferation, differentiation, and angiogenesis [19]. They are widely distributed in normal and tumor tissues, playing a useful role in tumor imaging (sst-scintigraphy or octreotide scan) [20]. More importantly, synthetic SST analogues (SSAs) represent a valuable therapeutic tool to treat ssts-positive tumors, to control hormone hypersecretion and tumor growth [21–24]. Additionally, ghrelin is a peptide hormone with multiple, and generally stimulatory functions, which span from hormone release to regulation of tumor cell proliferation [25,26]. Ghrelin needs to be acylated by the enzyme ghrelin-O-acyl transferase (GOAT) [27,28] to bind its receptor GHSR-1a [29]. The complexity of SST/CORT and ghrelin systems has been lately expanded by the identification of additional splicing variants of ghrelin (In1-ghrelin) [30–35], ghrelin receptor (GHSR-1b) [36,37] and sst5 (sst5TMD4 and sst5TMD5) [38–44] genes, which are overexpressed in tumoral pathologies, including gastroenteropancreatic NETs (GEP-NETs 34, 44), where they are associated with aggressive features.

The data collected in a limited number of studies on the presence and clinical implications of certain components of these systems [34,45–54] suggest that a more detailed knowledge of the expression pattern of their components could unveil relevant implications in the diagnosis, prognosis and medical treatment of LCs. Accordingly, we implemented an exhaustive characterization of the presence/expression of the components of SST/CORT and ghrelin systems in well-characterized LCs, compared to non-tumoral adjacent tissues and normal non-neoplastic samples, and explored their putative relationship with clinical/histological characteristics.

2. Materials and methods

2.1. Patients

The Ethics Committee of the Reina Sofia University Hospital (Cordoba, Spain) approved the study, which was conducted in accordance with the Declaration of Helsinki and with national and international guidelines. A written informed consent was signed by every individual. Seventy-five patients with LCs who underwent surgery from 2005 to 2015 were included. Clinical records were used to collect full medical history. Other LNETs such as LCNEC and SCLC were not included due to limitations in the number of available samples; while endocrine-associated syndromes such as MEN or von Hippel-Lindau syndromes were excluded. LCs were evaluated and classified according to histopathology features in TC and AC. To confirm the neuroendocrine nature of all tumors, different neuroendocrine markers (including chromogranin A, synaptophysin, cytokeratin 7, cytokeratin 20, CD56, neuronal specific enolase, AE1AE3, cytokeratin, p53, glucagon, insulin, gastrin, SST, intestinal vasoactive peptide, pancreatic polypeptide and/or serotonin) were determined by immunohistochemistry following standardized protocols and evaluated by two experienced pathologists to confirm that all the included neuroendocrine tumors expressed a minimum of two different neuroendocrine markers. In addition, tissue samples were obtained from 46 of those patients and from 7 normal tissues from anonymous body organ donors. In particular, we obtained 89 formalin-fixed paraffin-embedded (FFPE) samples (46 primary tumors and 43 non-tumoral adjacent tissues) and 7 normal tissues. To ensure the appropriate identification of tumor and non-tumor adjacent areas for further RNA isolation, a comprehensive analysis of hematoxylin/eosin and immunohistochemistry sections was performed by two different experienced pathologists using conventional microscopy. Each sample was evaluated twice in order to identify, delineate and manually dissect the corresponding tissues, and when tumor and adjacent tissue were appropriately identified, 5um slides from each paraffin-embedded tissue were cut and tumor and non-tumor adjacent regions subsequently separated for further evaluations.

2.2. RNA isolation and reverse-transcription

Total RNA from FFPE samples was isolated using the RNeasy FFPE Kit (Qiagen, Limburg, Netherlands) according to manufacturer's instructions. Quantification of the recovered RNA was assessed using NanoDrop2000 spectrophotometer (Thermo Scientific, Wilmington, NC, USA). One microgram of total RNA was retrotranscribed to cDNA with the First Strand Synthesis kit using random hexamer primers (Thermo Scientific) as previously reported [39,55].

2.3. Quantitative real time PCR (qPCR)

cDNAs were amplified with the Brilliant III SYBR Green Master Mix (Stratagene, La Jolla, CA, USA) using the Stratagene Mx3000p system and specific primers for each transcript of interest, as previously reported. Expression levels (absolute mRNA copy number/50 ng of sample) of native-ghrelin, In1-ghrelin, GOAT, GHSR-1a, GHSR-1b, SST, CORT, sst1, sst2, sst3, sst4, sst5 and sst5TMD4 were measured using previously validated primers [31,33,56,57]. Samples were run, in the same plate, against a standard curve to estimate mRNA copy number and a No-RT sample as negative control. Thermal profile consisted of an initial step at 95 °C for 30 s, followed by 50 cycles of denaturation (95 °C for 20 s) and annealing/elongation (60 °C for 20 s), and finally, a dissociation cycle (melting curve: 55 °C–95 °C, increasing 0.5 °C/30 s) to verify that only one product was amplified. RNA expression was adjusted by the expression of the housekeeping gen beta-actin (ACTB), whose levels were not significantly different among groups

2.4. Immunohistochemistry (IHC) analysis

IHC analysis of GHSR-1a and sst4 was implemented in formalin-fixed, paraffin-embedded (FFPE) lung tissue samples (n = 19), which included tumor and non-tumor regions from patients diagnosed with LCs, using standard procedures. The optimum antibody concentration to perform GHSR-1a and sst4 IHC analyses (1:300) using a commercially available human GHSR-1a and sst4 antibodies (Santa Cruz and AVIVA, respectively) was selected by performing a series of antibody dilution tests (1:100; 1:200; 1:300 and 1:400) in brain samples (a tissue that has been previously reported to express high levels of GHSR-1a and sst4). Two independent pathologists performed the IHC analysis of the samples following a blinded protocol. In the analysis, 1+, 2+, 3+ stand for low, moderate, and high intensities of the tumor region staining compared to the adjacent region with non-tumor lung tissue.

2.5. Statistical analysis

Paired *t*-test analysis was used to compare the expression levels between LC samples and adjacent non-tumor tissue. Non-paired *t*-test analysis was used to compare the expression levels between normal lung tissue and tumor or adjacent non-tumor tissue. U-Mann Whitney test was used to evaluate clinical-molecular relations. Chi-squared test compared categorical data. Statistical analyses were performed using SPSS statistical software v20 and GraphPad Prism v6. Data are expressed as mean ± SEM. *p*-values < 0.05 were considered statistically significant.

3. Results

3.1. Patient population and clinical correlations

A total of 75 LCs patients were included. Demographic and clinical features are summarized in Table 1, while characteristics of the 49 tumor tissues are summarized in Table 2. Demographic and clinical characteristics were similar between TC and AC subjects. When all LC patients were considered together, age was positively correlated to second neoplasm presence (p = 0.006) and parenchyma localization (p = 0.013), and showed a non-significant trend to correlate with vascular invasion (p = 0.055). Although all tumors were positive for at least one neuroendocrine marker, these markers did not exhibit any apparent association with clinical variables. In contrast, tumor diameter was directly correlated to necrosis (p = 0.016), peritumoral

Table 2
Tumor sample characteristics.

Tissue samples	Total (%) (46)	Typical n = 22(66.7%)	Atypical n = 11(33.3%)	p*
Primary tumor localization				
Right lung	62.2% (28)	72.7% (16)	54.5% (6)	> 0.05
Left lung	37.8% (17)	27.3% (5)	45.5% (5)	> 0.05
Upper lobe	25.6% (11)	18.2% (4)	36.4% (4)	> 0.05
Right middle lobe	27.9% (12)	40.9% (9)	18.2% (2)	> 0.05
Lower lobe	44.2% (20)	36.4% (8)	36.4% (4)	> 0.05
Immunohistochemistry				
Chromogranin A	39.6% (19)	34,28% (12)	40% (6)	> 0.05
Synaptophysin	31.3% (15)	28.57% (10)	28.57% (4)	> 0.05
Cytokeratin 7	4.2% [2]	2.86% (1)	9.1% (1)	> 0.05
Cytokeratin 20	2.1% (1)	0% (0)	7.14% (1)	> 0.05
Neuronal specific enolase	22.9% (11)	17.14% (6)	21.43% (3)	> 0.05
CD56	18.8% (9)	18.2% (4)	18.2% (2)	> 0.05
Others**	73% (36)	62.85% (22)	71.42% (10)	> 0.05
Functionality	4.3% (2)	0% (0)	8.3% (1)	> 0.05
Tumor diameter (cm)	**2.72 ± 2.05**	**2.38 ± 0.21**	**4.81 ± 1.36**	**0.013**
Multiple tumors	7% (5)	3% (1)	13.3% (2)	> 0.05
Peri-tumoral invasion	**22.5% (9)**	**6.7% (2)**	**46.2% (6)**	**0.028**
Vascular invasion	**16.7% (4)**	**0% (0)**	**50% (4)**	**0.005**
Neural invasion	11.8% (2)	0% (0)	33.2% (2)	> 0.05
Metastasis	**12.5% (5)**	**0% (0)**	**46.2% (5)**	**0,007**
Bronchial lumen localization/ infiltration	80% (32)	75% (21)	66.7% (8)	> 0.05
Parenchyma localization/ infiltration	32.5% (13)	35.7% (10)	58.3% (7)	> 0.05
Pleural localization/ infiltration	7.5% (3)	10.7% (3)	0% (0)	> 0.05

* *p* value refers to the comparison between typical and atypical carcinoids.
** Others indicate positive immunohistochemistry for (at least) one of the following neuroendocrine markers: AE1AE3, cytokeratin, p53, glucagon, insulin, gastrin, SST, intestinal vasoactive peptide, pancreatic polypeptide and/or serotonin.

Table 1
Demographic and clinical characteristics of the patient population.

General characteristic	Total n = 75(100%)	Typical n = 34(69.4%)	Atypical n = 15(30.6%)	p*
Gender				> 0.05
Male	48% (36)	44.1% (15)	66.7% (10)	
Female	52% (39)	55.9% (19)	33,3% (5)	
Age	53.13 ± 15.18 years	49.36 ± 3.45	51.66 ± 4.62	> 0.05
Personal history of other tumors	17.4% (12)	22.6% (7)	20% (3)	> 0.05
Smoke habit				> 0.05
Active	34% (18)	33.3% (8)	28.6% (4)	
Ex-smoker	28.3% (15)	33.3% (8)	28.6% (4)	
No habit	37.7% (20)	33.3% (8)	28.6% (4)	
Family history of neoplasms	55.6% (5)	66.7% (2)	33.3% (1)	> 0.05
Incidental tumor	21.7% (10)	29.4% (5)	36.4% (4)	> 0.05
Pre-surgical treatment	6.1% (4)	3.2% (1)	14.3% (2)	> 0.05
Clinical symptoms**				> 0.05
Hemoptysis	17.9% (5)	23.5% (4)	9.1% (1)	
Cough	10.7% (3)	11.8% (2)	9.1% (1)	
Pneumonia	35.7% (10)	35.3% (6)	36.4% (4)	

* *p* value refers to the comparison between typical and atypical carcinoids.
** Most common clinical symptoms.

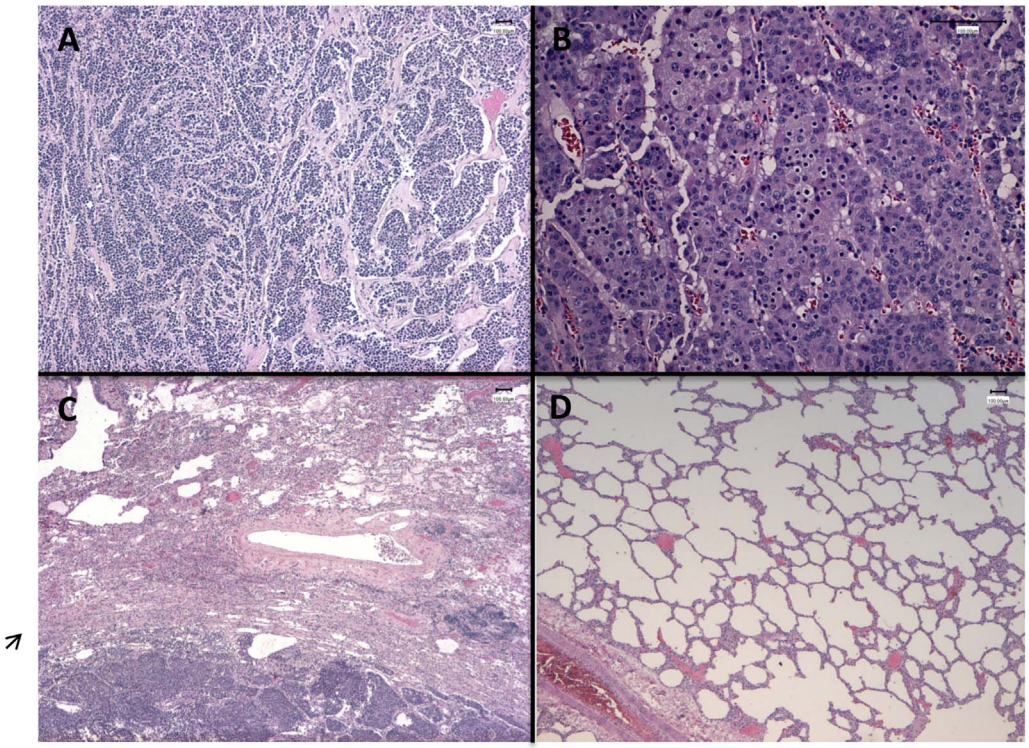

Fig. 1. Histopathological evaluation of normal lung, adjacent non-tumoral tissue and LCs samples. Representative images of hematoxilin/eosin staining performed on TC (A), AC (B), adjacent non-tumoral tissue characterized by diffuse interstitial chronic inflammation with lymphocytes, scattered plasma cells and occasional multinucleated giant cells (narrows) (C) and normal lung controls (D).

invasion ($p = 0.006$) and metastasis ($p = 0.026$). ACs exhibited significantly higher diameter ($p = 0.022$), necrosis ($p = 0.013$), vascular invasion ($p = 0.005$), peritumoral tissue invasion ($p = 0.028$), and metastasis ($p < 0.001$) than TCs.

3.2. Histopathological characterization of LCs and non-tumor adjacent tissue

Representative histological images of TC and ACs, adjacent non-tumor tissues and normal lungs are depicted in Fig. 1. Remarkably, adjacent non-tumor tissue displayed clear signs of pathological transformation (Fig. 1C) as they presented diffuse interstitial chronic inflammation characterized by lymphocytes, scattered plasma cells and occasional multinucleated giant cells, which was not observed in normal non-neoplastic lung tissues (Fig. 1D).

3.3. Expression of SST/CORT system components in control and LC samples

SST/CORT system components were expressed in a modest proportion of normal lung samples, as determined by qPCR. Only SST and sst3 were expressed in almost 50% (3 out of 7) of normal samples, whereas other SST/CORT components were only detected in 1 or 2 samples (Fig. 2A). In contrast, a high proportion of tumor and, also, adjacent non-tumor tissues expressed most of the SST/CORT system components (Fig. 2A). Specifically, SST, sst1, sst2, sst3, sst5, and sst5TMD4 were expressed in at least 75% of adjacent non-tumor and tumor samples, with only sst4 being present in less than 25% of the adjacent and tumor tissues (Fig. 2A). Of note, the percentage of tumor tissues expressing the components of SST/CORT system was similar between AC and TC ($p > 0.05$) (Suppl. Fig. 1).

Expression levels of the SST/CORT system components were largely variable. In this analysis, only cases that showed detectable expression were included. SST expression levels were 100-fold higher than those of CORT, whose mRNA levels were close to the detection limits (Fig. 2B). SST levels were higher in non-tumor adjacent and tumor tissues compared to control tissue, being this increase more pronounced in tumor tissue (Fig. 2B). In the case of the receptors, sst1 and sst2 were highly expressed, followed by sst3, while sst5, sst5TMD4 and sst4 showed lower levels. Expression of all ssts, except sst3, displayed a similar tendency, increasing progressively from control tissues to non-tumor adjacent tissue and being apparently higher in the tumor regions (Fig. 2B). Interestingly, in tumor samples, SST expression was correlated to sst1, sst2, sst3 and sst5 expression; CORT levels were correlated to sst5 expression and, finally, sst1, sst2 and sst3 expression levels showed significant correlations (Suppl. Table 1).

3.4. Expression of ghrelin system components in control and LC samples

Ghrelin system components were also expressed in < 25% of normal lung samples, as determined by qPCR (Fig. 3A). In contrast, ghrelin, In1-ghrelin, GHSR-1a and GHSR-1b were expressed in at least 75% of tumor and adjacent non-tumor tissues; while GOAT was present in less than 50% of the adjacent non-tumor samples but in more than 75% of tumor samples (Fig. 3A). The proportion of tumor tissues expressing ghrelin system components was not statistically significant different between AC and TC (Suppl. Fig. 2). In contrast, GHSR-1a and GHSR-1b were overexpressed in tumor tissue and adjacent-non tumor tissue compared to normal lung tissue (Fig. 3B). No significant correlation was observed among the expression levels of the ghrelin system components in tumor samples (data not shown).

3.5. Immunohistochemistry (IHC) analysis

Although qPCR is a sensitive method of assaying for gene expression, we subsequently performed IHC analysis in a set of selected samples in order to validate the observed changes at the protein level, and to determine which particular cells are expressing those markers. To this end, we selected sst4 and GHSR-1a due to their clear overexpression in tumor samples. Specifically, GHSR-1a and sst4 IHC was

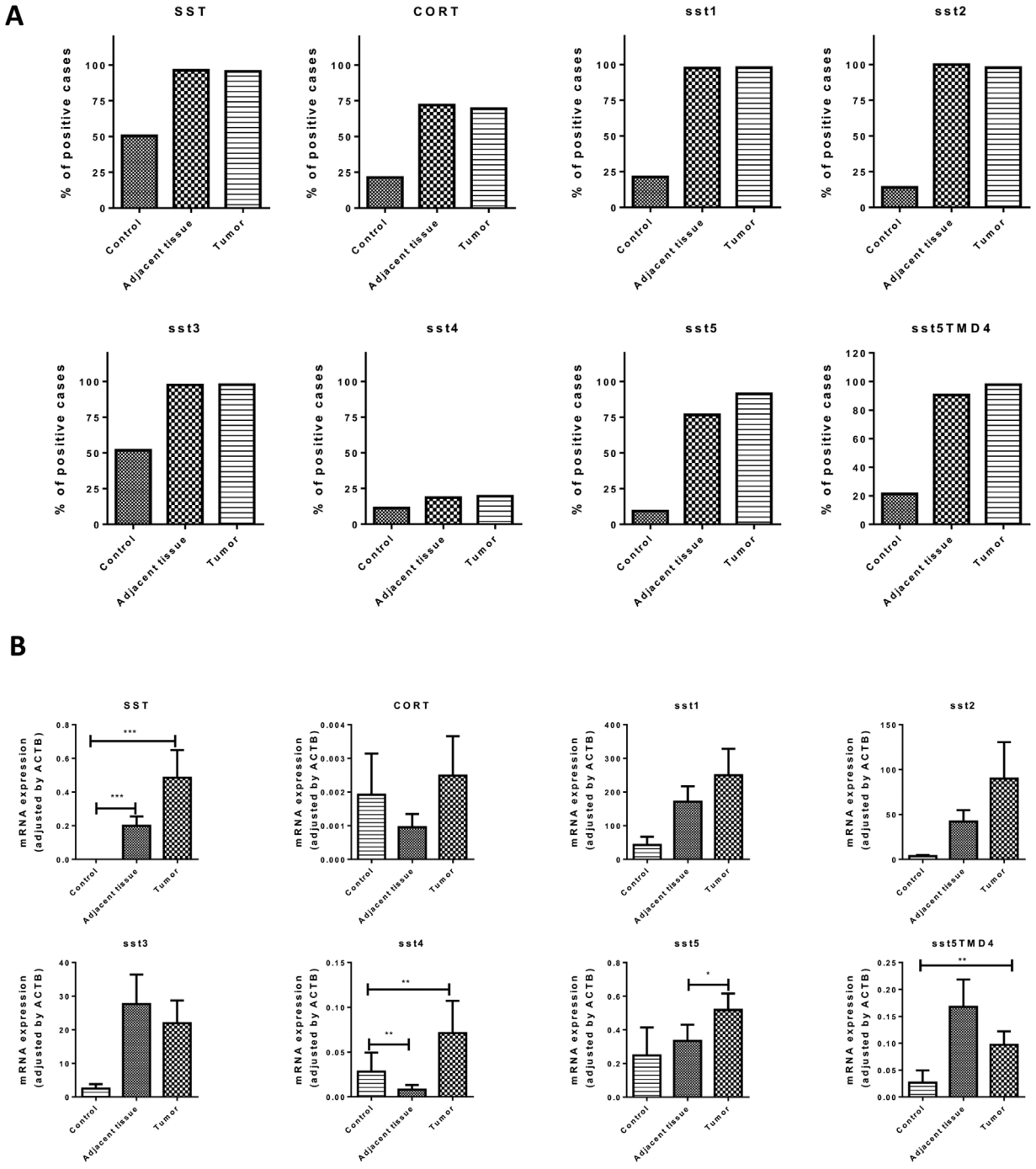

Fig. 2. Presence and mRNA expression of SST/CORT system components in normal lung, adjacent non-tumoral tissue and LCs. A: The graphs indicate the percentage of samples (normal lung control, adjacent non-tumoral tissue and tumoral tissue) positive for each of the SST/CORT system components. B: The absolute mRNA expression of the different components of the SST/CORT system was determined by qPCR in normal lung controls, adjacent non-tumoral tissue and LC samples (values are adjusted by ACTB expression). Data represent the mean ± SEM. Asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) indicate significant differences by paired analysis between adjacent non-tumoral and LCs and non-paired analysis between normal lung tissue and adjacent non-tumoral or tumoral tissues.

performed on FFPE-lung carcinoids, which revealed stronger staining in tumor samples compared to non-tumor adjacent tissue (Suppl. Figs. 3B and 4B). In general, IHC analysis of non-tumor adjacent tissue revealed that both the number of cells and the intensity of the staining was particularly low in normal airway epithelium and associated neuroendocrine cells, as well as in pulmonary parenchyma and associated glandular tissue (Suppl. Figs. 3A and 4A). However, it is worth noting that infiltrated immune cells and especially alveolar macrophages presented an intense staining in these samples. In contrast, IHC analysis of tumor tissue revealed that GHSR-1a and sst4 were present in the vast majority of tumor cells. Interestingly, tumor samples presented variable levels of both molecules, although most samples were classified as having an intensity of 2+ or 3+ by the pathologists. Therefore, these data confirm the contention that the expression of certain components of the SST/CORT/ssts and ghrelin/GHSRs systems, and especially GHSR-1a and sst4, is clearly dysregulated in LC samples compared to non-tumor adjacent tissue.

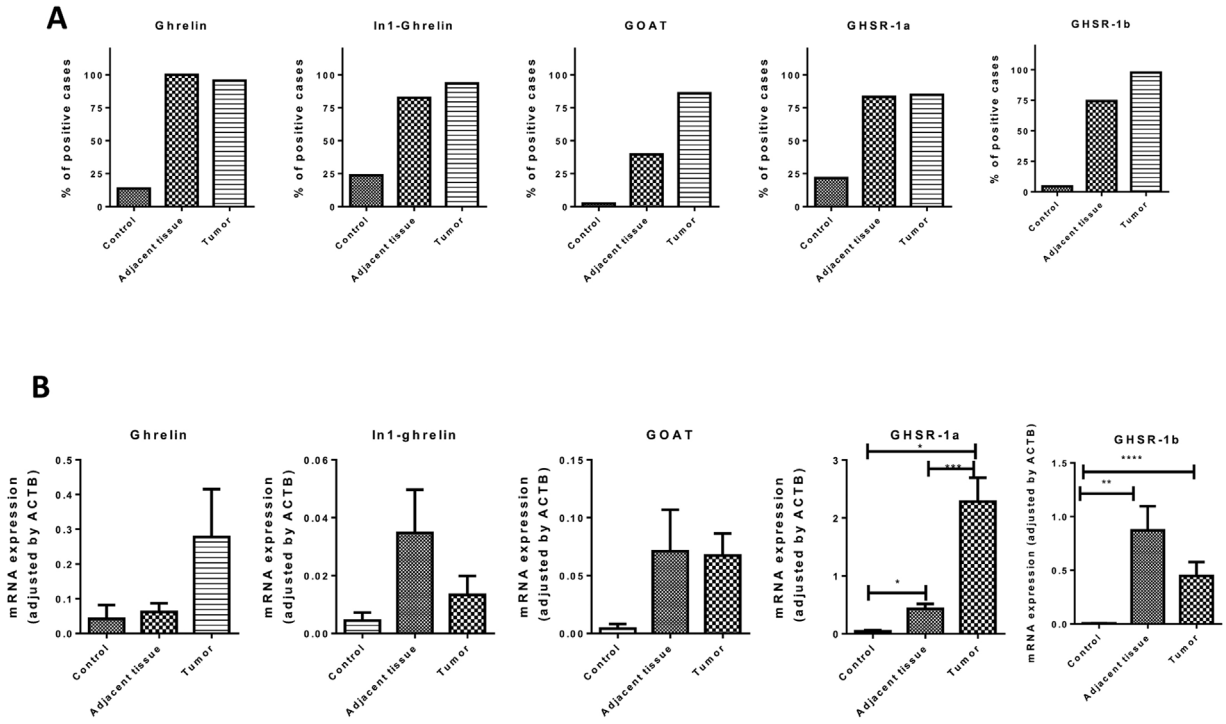

Fig. 3. Presence and mRNA expression of ghrelin system components in normal lung, adjacent non-tumoral tissue and LCs. A: The graphs indicate the percentage of samples (normal lung control, adjacent non-tumoral tissue and tumoral tissue) positive for each of the ghrelin system components. B: The absolute mRNA expression of the different components of the ghrelin system was determined by qPCR in normal lung controls, adjacent non-tumoral tissue and LC samples (values are adjusted by ACTB expression). Data represent the mean ± SEM. Asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) indicate significant changes by paired analysis between adjacent non-tumoral and LCs and non-paired analysis between normal lung tissue and adjacent non-tumoral or tumoral tissues.

3.6. *Expression of SST/CORT and ghrelin systems components and clinical-histological characteristics in LC samples*

Correlation analyses revealed that sst3 was overexpressed in LCs of patients with tobacco smoke exposure ($p < 0.05$), that sst5 was higher in incidental tumors, and that disease-free patients exhibited higher sst5TMD4 expression (Fig. 4). Regarding the ghrelin system, ghrelin expression was correlated to vascular invasion ($p = 0.042$) and tended to associate with bronchial localization ($p = 0.057$) (Fig. 4). Interestingly, necrotic tumors overexpressed GOAT ($p < 0.05$) and GHSR-1a was overexpressed in tumors with parenchyma localization and in non-functional and metastatic tumors ($p < 0.05$) (Fig. 4).

4. Discussion

In this study, we have comprehensively evaluated by qPCR the expression of SST/CORT and ghrelin system components in a large series of well-characterized TCs and ACs, and compared with the expression in adjacent non-tumor and normal lung tissues. To the best of our knowledge, this study represents the first systematic characterization of the components of these regulatory systems in samples from LCs, in comparison with adjacent non-tumor regions and normal lungs, and may therefore provide a useful overall picture of the landscape of changes associated to LCs pathology. Although several studies have explored the presence of certain SST/CORT and ghrelin systems components in LCs [45–52], their presence had never been compared to that found in adjacent non-tumor regions or normal lungs. In addition, specific SST/CORT and ghrelin system components displayed clinical-histological correlations in tumor tissues, suggesting that they could provide novel, valuable markers for LC patient management.

Consistent with previous reports showing that 75% of LCs are central (bronchial) and 25% are peripheral tumors [3], our tumor series presented a similar distribution. In addition, previous studies have reported that LCs diameter is not correlated to survival or recurrence [13]. Consistently, in our series, no direct correlation with clinical outcome was observed, although tumors > 2.4 cm showed higher rate of peritumoral invasion, vascular invasion and distant metastasis. In fact, no correlations between clinical, histological or immunohistochemical characteristics and survival or mortality were found herein, which is in contrast with previous reports describing some independent predictors of survival [mitotic rate, tumor size, sex [58], typical histology and lymphatic invasion [59]. Nevertheless, our analysis revealed that age correlated directly to the presence of parenchyma localization, a second neoplasm and vascular invasion, which have not been previously reported and could suggest age as a risk factor for more aggressive tumors.

Remarkably, tumor characteristics of TCs and ACs were markedly different. ACs exhibited significantly higher diameter, necrosis, *peri*-tumoral and vascular invasion and metastasis, which is consistent with higher ACs malignancy compared to TCs [60]. Similar to other reports, adjacent non-tumor tissues exhibited signs of pathological alteration compared to normal lung samples [61–64]. Consequently, to comprehensively characterize SST/CORT and ghrelin system components expression in a large series of well-characterized TCs and ACs, and compare with their expression in adjacent non-tumor tissues and normal lungs, we applied a qPCR-based approach as previous studies have demonstrated that mRNA levels of the components of these systems correlate well with their respective protein levels [46,48,49,65,66]. Moreover, qPCR is a more sensitive detecting method than IHC [67].

NETs are known to overexpress stss [46,53,54], which is important in their diagnosis and management [20,46,68,69]. However, to date, only a limited number of studies have reported the expression of ssts other than sst2, with variable results, likely due to the application of different experimental approaches [19,70,71]. Particularly, just few studies have explored a small number of cases or only single receptor subtypes [50,65,67,70,72]. Recently, additional studies have more comprehensively characterized ssts presence on LCs [46,48,73]. Inter-

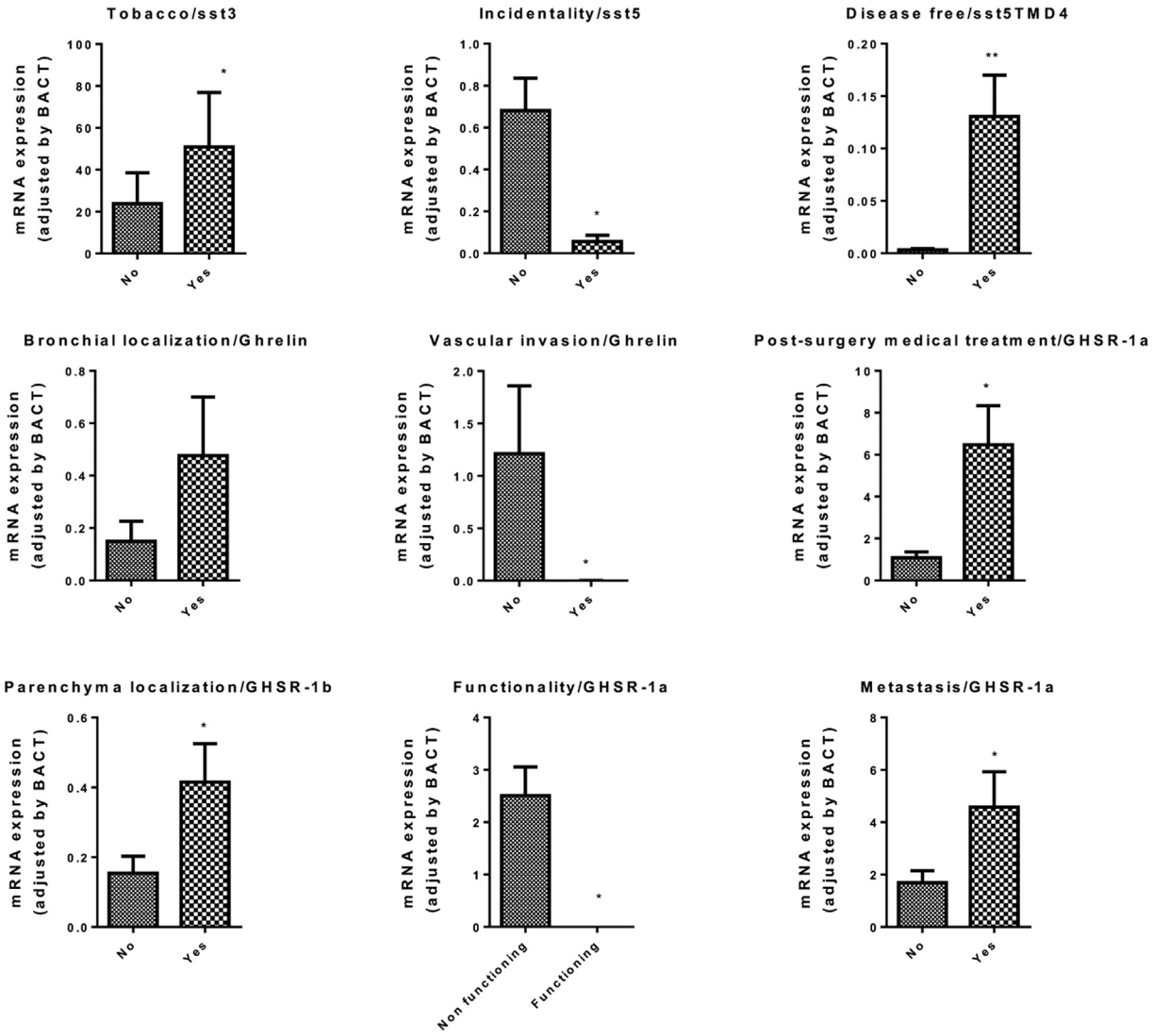

Fig. 4. Correlations between epidemiological, clinical, histological and molecular parameters in LCs. The putative correlations between epidemiological, clinical, histological and molecular parameters within LC samples were assessed by U-Mann Whitney tests and asterisks (*, $p < 0.05$; **, $p < 0.01$) indicate significant associations.

estingly, the data presented in the current study reveal the prominent and widespread expression of ssts in LCs, being sst1 the most abundant, followed by sst2, sst3 and sst5, with sst4 and the truncated sst5TMD4 being the least expressed. These data agree with the majority of the previous studies [48–52,65,67]. Moreover, this is the first study reporting the presence of sst5TMD4 in LCs and sst5 in symptomatic patients. Although initial studies suggested that ssts presence in LNETs could exhibit a progressive decrease from low- to high-grade forms [45]; however, our work and other studies [46] indicate that there are no major differences, suggesting that ssts could be a common hallmark of low and intermediate grade LNETs. Moreover, our results revealed, for first time, a prominent expression of SST in LCs, whereas CORT expression was comparatively negligible. Of note, expression of SST directly correlated with that of sst1, sst2, sst3 and sst5, which suggests an autocrine/paracrine SST/ssts loop capable to modulate *in situ* the progression of LCs. In addition, SST and ssts expressions displayed herein a gradation in normal lungs, non-tumor adjacent tissue, and LCs. Of special interest is our observation that non-tumor adjacent tissues also present a notable expression of SST and ssts, and in fact, a similar proportion of non-tumor adjacent tissues presented ssts compared to LCs, although, in general, at lower expression levels. To further explore this notion, we selected sst4 (due to its differential distribution), to perform an IHC analysis, as it has been previously reported that specific antibodies against SST receptors allow an appropriate immunolocalization of receptor subtypes in tumor tissue with a comparable, although not quantitatively superior quality than that of qPCR [65]. Results from this analysis enabled visualization of sst4 in specific cells of tumor tissue and demonstrated that non-tumor adjacent cell types (in airway epithelium and associated neuroendocrine cells, as well as in pulmonary parenchyma and associated glandular tissue) are less immunopositive than tumor cells.

Our results also revealed a differential expression of ghrelin system components in normal lung tissues, non-tumor adjacent tissue, and LCs. In particular, ghrelin system components were expressed at low levels in a reduced proportion of normal lungs, which is consistent with previous reports showing ghrelin expression in normal and fetal lungs [30,47,66,74]; while GHSR1a was undetectable [74]. In contrast, our analysis revealed a prominent and widespread expression of ghrelin system components in LCs and adjacent non-tumor samples. Interestingly, a higher expression levels of the canonical variants (native ghrelin and GHSR1a) is consistent with previous reports showing that ghrelin is expressed in lung tumors, regardless of their neuroendocrine phenotype, and that GHSR1a is present in well differentiated functioning and non-functioning lung NETs [29,75]. This was further supported by IHC analysis of GHSR1a, which demonstrated higher staining of LC cells compared to the rest of non-tumor adjacent cell types (in airway

epithelium and associated neuroendocrine cells, as well as in pulmonary parenchyma and associated glandular tissue). In contrast, expression of the alternative splicing variants (In1-ghrelin/GHSR1b) is lower, and had not been reported previously. This is also the first study reporting the expression of the GOAT enzyme in a high proportion of LCs, wherein the concomitant presence of ghrelin, GOAT and GHSR1a on most LCs suggests the existence of a functional regulatory association that could be modulating the development and/or progression of this pathology. Unfortunately, no studies have yet investigated the direct effect of this in LCs, and the only report in SCLC suggests that ghrelin could inhibits cell proliferation and increases apoptosis [29], in agreement with the negative association between ghrelin and vascular invasion in our cohort. However, GOAT levels were higher in tumors with necrosis, which were the ones with a larger size and higher capacity of peritumoral invasion and distant metastasis; this, together with the direct relationship between metastasis, requirements of post-surgical treatments, and GHSR1a expression, reinforces the idea that this system could be associated to the pathogenesis of the disease and might therefore provide novel potential diagnostic, prognostic and/or therapeutic tools in LCs.

In summary, this study provides a comprehensive primary mapping of the expression of SST/CORT and ghrelin system components (including their most relevant splicing variants), in LCs, as compared with their respective adjacent non-tumoral tissues, and with normal, non-neoplastic tissues. Our results indicate a prominent and widespread overexpression of SST/CORT and ghrelin system components in LCs and in non-tumoral adjacent tissues, wherein they could exert relevant regulatory roles, for they display changes in expression tightly linked to the degree of disease, and exhibit associations to fundamental clinical parameters. Hitherto, there has been a paucity of studies reporting clinical, biochemical, histological, immunohistochemical or molecular tumor markers that could help to accurately predict the efficacy of the medical treatment, as well as the cure or relapse rates in NETS. This goal is specially difficult and necessary in LCs, due to their rarity, high diversity and heterogeneity in terms of malignant capacity, localization, and growth pattern. In this context, our present findings may help to identify new potential diagnostic and prognostic factors, which could help to devise and implement improved therapeutic strategies, aimed at attaining a better quality of life and survival for this patients. Hence, our data provide novel information on the presence of both SST/CORT and ghrelin systems in LCs, and invite to suggest that their role in this pathology as putative molecular biomarkers and therapeutic targets for LC patients deserves further investigation.

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Conflict of interest

The authors have no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.lungcan.2017.05.006.

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