Expression of Somatostatin Receptors 1–5 and Dopamine Receptor 2 in Lung Carcinoids: Implications for a Therapeutic Role

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Key Words

Somatostatin receptor subtypes · Dopamine receptor 2 · Immunohistochemistry · Lung carcinoids

Abstract

Objective: The expression of somatostatin receptors (SSTRs) and dopamine receptor 2 (DR2) in neuroendocrine tumors is of clinical importance as somatostatin analogues and dopamine agonists can be used for their localization and/or treatment. The objective of this study is to examine the expression of the five SSTR subtypes and DR2 in lung carcinoids (LCs). **Methods:** We conducted a retrospective study of 119 LCs from 106 patients [typical carcinoids (TCs): n = 100, and atypical carcinoids (ACs): n = 19]. The expression of all five SSTR subtypes and DR2 was evaluated immunohistochemically and correlated to clinicopathological data. In a subgroup of cases, receptor expression was further analyzed using semiquantitative RT-PCR. **Results:** SSTR2A was the SSTR subtype most frequently expressed immunohistochemically (72%), followed by SSTR1 (63%), SSTR5 (40%), and SSTR3 (20%), whereas SSTR4 was negative. DR2 was expressed in 74% and co-expressed with SSTR1 in 56%, with SSTR2A

in 59%, with SSTR3 in 19%, and with SSTR5 in 37% of the tumors. Receptor expression was not related to the histological subtype, tumor aggressiveness (disease extent/grading) or functionality; however, DR2 was expressed more frequently in ACs than TCs (95 vs. 70%, p = 0.017). In a subset of patients, RT-PCR findings highly suggested that the expression of SSTR2A, SSTR3, DR2, and to a lesser extent that of SSTR1 and SSTR5 is the outcome of increased gene transcription. **Conclusions:** The high and variable immunohistochemical expression of the majority of SSTRs along with their co-expression with DR2 in LCs provides a rationale for their possible treatment with agents that target these receptors.

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Introduction

The respiratory tract comprises one of the most common primary locations of neuroendocrine tumors (NETs; 20–30%) that have an incidence of 1.35 cases per 100,000 inhabitants [1, 2]. NETs of the respiratory tract are divided into well-differentiated (lung carcinoids, LCs) and poorly differentiated endocrine carcinomas (PDECs).

According to the WHO 2004 classification, LCs are further subdivided into typical carcinoids (TCs) and atypical carcinoids (ACs). TCs are characterized by <2 mitoses/10 high-power fields (HPF) and the absence of necrosis, whereas ACs are defined by 2–10 mitoses/10 HPF and/or the presence of necrosis. PDECs are distinguished by the presence of >10 mitoses/10 HPF and necroses and are subdivided further into large-cell neuroendocrine carcinomas (LCNEC) and small-cell lung carcinomas (SCLC) according to their morphological features [3, 4].

Somatostatin receptor (SSTR) expression is of importance in the diagnosis and management of patients with NETs. The SSTR subtypes 1–5 are targeted via somatostatin receptor scintigraphy (SRS) and long-acting somatostatin analogues (SAs) [5, 6] and/or peptide receptor radionuclide treatment [7] for diagnostic and therapeutic purposes, respectively. To date, only a limited number of studies have demonstrated the expression of SSTR subtypes other than SSTR2A in NETs with divergent results, possibly due to differences in the methodology applied [8–18]. Data concerning LCs are even more sparse and inconsistent [15, 18–23]. Among the five subtypes of SSTRs, SSTR2A is the one most frequently expressed, exhibiting mainly membranous immunostaining, while the other subtypes present mainly cytoplasmic expression [24]. Recently, the precise expression of SSTRs in NETs has become more relevant as newer SAs with an affinity to multiple SSTRs have become available [25].

Five dopamine receptor (DR) subtypes have been cloned and they are divided further into D1-like and D2-like. Among DRs, DR2 is of particular importance since it is the main target for dopamine agonists (DAs) [26]. This receptor exists in two isoforms: D2-long and D2-short and is expressed in a variety of NETs. There is evidence to suggest a synergistic action between SAs and DAs in controlling hormonal secretion from functioning NETs [27]. This may be attributed to heterodimerization between SSTRs and DRs that result in new oligomeric receptor entities with enhanced functional activity [28]. To date, only few studies have investigated the expression of DR2 in NETs, and only two included a small number of LCs [11, 29–32]. In addition, the co-expression of SSTRs and DRs has not been examined in LCs.

The purpose of this study is to investigate the expression of the five SSTR subtypes along with DR2 in LCs by means of immunohistochemistry (IHC) and compare their expression to real-time polymerase chain reaction (RT-PCR) and SRS findings of the same patients in order to provide a rationale for possible treatment with agents that target these receptors.

Materials and Methods

Patients and Tumors

This study included 119 primary LC tumors as well as lymph node (n = 11) and distant metastases (n = 6) derived from 106 patients (63 females, 59%) from three institutions. Six patients harbored two tumors, whereas in 3 patients, the disease consisted of multiple tumorlets. The mean age at diagnosis was 52.0 ± 15.4 years (range 16–82). In 2 patients, LCs emerged in the context of multiple endocrine neoplasia 1 (MEN 1) syndrome. Six patients had received chemotherapy and/or biotherapy preoperatively: paraplirin and vepecid (n = 1), temozolomide (n = 3) and interferon- α (n = 2). Two patients had received SAs in the context of carcinoid syndrome.

According to the WHO 2004 criteria, the tumors were classified as TCs (n = 100) and ACs (n = 19). The mean primary tumor diameter was 22.3 ± 12.4 mm. Six patients presented with symptoms of hormonal hypersecretion (functioning tumors); 3 with Cushing's syndrome due to ectopic adrenocorticotrophin secretion, and 2 with typical and 1 with atypical carcinoid syndrome. At the time of diagnosis, 19 patients (21%; 13 TCs and 6 ACs) had lymph node involvement, whereas 12 patients (11%; 6 TCs and 6 ACs) had distant metastases. No significant difference was observed between TCs and ACs regarding patients' sex, age at initial diagnosis and functional status, whereas ACs had a significantly larger mean tumor size (table 1). For comparison reasons, the expression of the five SSTRs and DR2 were also studied in 20 PDECs: biopsy material of 12 SCLCs (10 pure and 2 combined with LCNEC) and 8 LCNECs (surgical material).

SRS data (obtained prior to tumor excision) were available in 18 patients. In 16 cases, SRS with 175–220 MBq ^{111}In -DTPA-octreotide was performed, and the results were evaluated 24 h after administration, according to Krenning's scale (KS; 0 = no abnormality; 1 = faint uptake in the tumor; 2 = clear uptake in the tumor but less than in the liver; 3 = uptake greater in the tumor than in the liver, and 4 = uptake much greater in the tumor than in the liver) [33]. In 2 cases, where only ^{68}Ga -DOTATOC PET scan was available, a SUV max >5.5 was considered as positive.

Controls

Macroscopically normal pancreatic tissue from 1 patient operated for a pancreatic NET was used as the 'positive' control for SSTRs. Normal adenohypophysis tissue obtained post-mortem as well as pituitary adenomas with documented response to DA were used as 'positive' controls for DR2. Normal human tonsil was used as the 'positive' control for the Ki67 labeling index (LI). 'Negative' immunostaining controls included omission of the primary antisera and replacement of the primary antibody by nonimmune serum at the same dilution as the primary antibody in question and in the same diluent. In occasional cases, a neutralization test was conducted following a 24-hour incubation of primary antiserum with the relevant antigen (10 nmol antigen per milliliter diluted antibody solution) before application to the sections. The SSTRs antigens used were from Gramsch Laboratories (Schwabhausen, Germany): SSTR1 (SS-841); SSTR2A (SS-801); SSTR3 (SS-851); SSTR4 (SS-881), and SSTR5 (SS-891) as well as from Epitomics Inc. (Burlingame, Calif., USA): SSTR2 (P-3582–1); the antigen for DR2 (NLS1403PEP) was from Novus Biologicals (Littleton, Colo., USA).

Table 1. Clinicopathological data of the cohort of 106 patients with LCs, overall and stratified according to tumor histology

	Overall (n = 106)	TCs (n = 91)	ACs (n = 15)	$\chi^2(t)$	p
Sex, female/male	63/43	53/38	10/5	0.379	0.585
Age at diagnosis, years	52.0±15.4	52.0±14.7	51.4±19.7	(0.150)	0.881
Size of primary tumor, mm	22.3±12.4	21.0±11.2	30.3±16.1	(2.777)	0.007
Functional status	6 (5.7)	4 (4.4)	2 (13.3)	2.881	0.155
Lymph node involvement	19 (21)	13 (14)	6 (40)	5.543	0.029
Distant metastases	12 (11)	6 (7)	6 (40)	12.090	0.003

Values are presented as $\pm$ SD or n (%) unless otherwise indicated.

Immunohistochemistry

The neuroendocrine differentiation of the tumors was confirmed by routine immunostaining with the general neuroendocrine markers chromogranin A and synaptophysin. Immunostaining was applied using a polymer detection system (Dako Cytomation, EnVision® + System-HRP, K4010). The Dako Autostainer was used for the routine immunostaining of the sections with the Ki67 antibody. Diaminobenzidine was used as chromogen. The sections were incubated with the antibodies overnight at room temperature. Before immunostaining, the sections were micro-wave treated for 2 × 5 min at 750 W using Tris buffer saline (pH 8.0) as retrieval solution.

The primary SSTRs and DR2 antibodies were polyclonal rabbit. The antibodies for the SSTRs were from Gramsch Laboratories: SSTR1 (SS-840, dilution 1:5,000); SSTR2A (SS-800, 1:5,000); SSTR3 (SS-850, 1:5,000); SSTR4 (SS-880, 1:100), and SSTR5 (SS-890, 1:5,000). The DR2 antibody (NLS1403, 1:750) was purchased from Novus Biologicals. In 115 of the primary LCs and in all PDECs, immunostaining was applied with the novel monoclonal rabbit anti-SSTR2A antibody, UMB-1 (1:200; Epitomics Inc.). Four primaries and all the specimens from lymph node or distant metastases were excluded due to insufficient tissue material. The Ki67 antibody used for the immunostaining of the tumor specimens was mouse monoclonal from Dako (Clone MIB-1, M7240, dilution 1:200).

Calculation of Immunoreactive Cells

In consecutive immunostained sections, the percentages of SSTR and DR2 immunoreactive cells were estimated by light microscopy at a magnification of ×400 using a square grid in one of the oculars. At least five randomly selected areas were examined; in smaller lesions the whole of the neoplastic tissue.

Tumors were considered 'positive' if a membrane pattern was observed in >10% of the tumor cells (a threshold shown to comply with autoradiography results) [34] and/or when cytoplasmic immunostaining was found in the majority (>50%) of the tumor cells. In addition, the localization of receptor expression was assessed quantitatively with the scoring system proposed by Volante et al. [35] as follows. Score 1 (S1): pure cytoplasmic immunoreactivity; score 2 (S2): a minority tumor cell population showed membranous immunoreactivity in <50% of the cell circumference, and

score 3 (S3): a majority tumor cell population revealed membranous immunoreactivity in over 50% of the circumference.

One investigator (L.G.) categorized the tumors into TCs, ACs and PDECs as well as determined the rate of cell proliferation by calculating the Ki67 LI. Mitoses were counted on hematoxylin and eosin-stained slides at 40 HPF. The Ki67 LI was assessed in at least 2,000 tumor cells in areas where the highest nuclear labeling was observed (hot spots). The LI was calculated as the percentage of Ki67 immunoreactive cells. The analysis of the SSTRs and DR2 immunostainings was done by two investigators (L.G. and A.V.T.), and when there was discordance a post-review unanimous decision was made.

Semiquantitative RT-PCR Analysis

In a subset of 20 LCs, the five SSTRs and DR2 gene transcripts were analyzed using semiquantitative RT-PCR. Total RNA extraction and subsequent cDNA synthesis with reverse transcription were performed using standard techniques. Amplification of cDNAs was achieved using ready-to-use Long-Range PCR Nucleotide Mix (Roche Diagnostics Corp., Indianapolis, Ind., USA) and appropriate temperature programs on a MJ Thermocycler (Bio-Rad). The data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels which served as internal control. The primer sequences for SSTR1, SSTR2A, SSTR3, SSTR4, SSTR5, DR2, and GAPDH (control gene) are listed in online supplementary table 1 (see www.karger.com/doi/10.1159/000381061).

Statistical Analysis

Tumors were stratified according to their size (largest dimension): ≤3 and >3 cm. This cutoff point was selected according to the TNM classification system proposed by the American Joint Committee on Cancer as it is the limit between T1 and T2 tumors (AJCC cancer staging manual ed 7, 2010). For nominal data, the χ^2 test was used. Cohen's kappa coefficient was used to assess the concordance between data and Spearman's *rho* (r_s) coefficient to indicate correlation among ordinal data. The level of statistical significance was set to 0.05. Calculations were performed using the Statistical Package for Social Sciences V.13.0 software (SPSS Inc., Chicago, Ill., USA).

The research protocol was reviewed and approved by the local research ethics boards (Uppsala University Hospital, Uppsala, Sweden, and University of Athens Medical School, Athens, Greece).

Fig. 1. Relative incidence of expression of the five SSTR subtypes and DR2 in TCs and ACs. SSTR2A results were obtained by the SS-800 antibody provided by Gramsch.

Results

Controls

Immunostaining with the antibodies against the five SSTR subtypes revealed distinct immunoreactive cells in the pancreatic islets. Similarly, positive cells were observed in the normal adenohypophysis tissue and pituitary adenomas when immunostained with the anti-DR2 antibody. No immunoreactivity was seen after the omission of the primary antiserum in question after its replacement by nonimmune serum or after the neutralization test.

Immunohistochemistry and Statistical Analysis

The vast majority of the tumor cells in the LCs expressed chromogranin A and synaptophysin. For all the SSTRs examined, positive tumor cells lying at the periphery had a tendency to express a membranous immunostaining pattern with a stronger intensity than those in the central part of the specimen. SSTR2A was the receptor most frequently expressed as it was detected in 90 cases (76%) by using the SS-800 antibody and in 80 cases (67%) by using the UMB-1 (mean 72%), followed by SSTR1 (75 cases, 63%), SSTR5 (48 cases, 40%) and SSTR3 (24 cases, 20%); SSTR4 was not detected in any of the tumors. Overall, 99 out of 119 tumors (83%) were positive for at least one SSTR subtype. DR2 was detected in 88 tumors (74%). No significant difference was observed in the expression of any SSTR subtype between TCs and ACs, whereas DR2 was expressed more frequently in ACs (95 vs. 70%, $p = 0.017$; fig. 1).

Regarding SSTR2A, the use of UMB-1 antibody revealed more tumor cells with membrane immunostaining pattern and with less cytoplasmic localization compared to the SS-800 antibody (fig. 2). In 35 cases, the immunostaining quality was improved in comparison to SS-800 as 12 tumors were reclassified as S2 and 23 tumors as S3. Conversely, 17 tumors were reconciled as negative with the UMB-1. However, when the two antibodies were compared to the RT-PCR and SRS results, the SS-800 performed better compared to the UMB-1 antibody (88 vs. 75% concordance and 90 vs. 71% concordance, respectively). Consequently, the results obtained with both antibodies were considered acceptable and both were reported unless stated otherwise.

Assessment of the receptor localization with the scoring system proposed by Volante et al. [35] revealed that the immunostaining pattern of SSTR1, SSTR3 and SSTR5 was mainly cytoplasmic [(S1: 65% vs. S2: 34% vs. S3: 1%), (S1: 88% vs. S2: 8% vs. S3: 4%) and (S1: 91% vs. S2: 7% vs. S3: 2%), respectively]. In contrast, SSTR2A exhibited a predominantly membranous (S2 or S3) pattern (S1: 10% vs. S2: 41% vs. S3: 49% for the UMB-1 and S1: 29% vs. S2: 46% vs. S3: 25% for the SS-800 antibody). The expression of DR2 was exclusively cytoplasmic (S1: 100%)

We also examined the distribution of SSTR subtypes in tumors negative for SSTR2A. Among 29 tumors negative to the SS-800 antibody, 14 (48%) expressed another SSTR. The corresponding figures were 10 (35%), 2 (11%) and 2 (11%) for SSTR1, SSTR3 and SSTR5, respectively. Among 39 tumors negative to the UMB-1 antibody, 29 (74%) in total were positive for SSTR1, SSTR3 or SSTR5 analyzed for each receptor as 17 (44%), 5 (13%) or 7 (18%), respectively.

SSTR2A, SSTR3 and SSTR5 exhibited selective immunostaining in the tumoral and nontumoral fibrovascular

Fig. 2. LCs immunostained for the five SSTR subtypes and DR2. **A** SSTR1: predominantly cytoplasmic and partially membranous immunostaining in the minority of the tumor cells (S2 according to Volante et al. [35]). **B1** SSTR2A (SS-800 antibody): circumferential membrane immunostaining in the majority of the tumor cells (S3 according to Volante et al. [35]), faint cytoplasmic staining is also observed. **B2** SSTR2A (UMB1 antibody): circumferential membrane immunostaining (S3), while cytoplasmic staining is almost absent. This photograph is taken from the same area of the same tumor as in **B1**. **C** SSTR3: S3 according to Volante et al. [35] and intense immunoreactivity at the fibrovascular stroma. **D** SSTR4 was nonimmunoreactive. **E** SSTR5: immunohistochemical pattern as in S2. **F** DR2: cytoplasmic immunostaining in virtually all tumor cells. **Insets** in each panel depict the neutralization test for the corresponding receptor. Scale bars = 100 μ m.

(For figure see next page.)

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Table 2. Association of the relative distribution of SSTRs 1–5 and DR2 with different indices of tumor aggressiveness (disease extent and grading) and tumor functionality

	PAD		SSTR1		SSTR2A ^a		UMB-1 ^b		SSTR3		SSTR5		DR2		Total
	TC	AC	+	–	+	–	+	–	+	–	+	–	+	–	
T _{any} N ₀ M ₀	67 (82)	8 (53)	47 (63)	28 (37)	56 (75)	19 (25)	52 (69)	23 (31)	14 (19)	61 (81)	32 (43)	43 (57)	53 (71)	22 (29)	75
T _{any} N _{1–3} M ₀	9 (11)	1 (7)	6 (60)	4 (40)	7 (70)	3 (30)	7 (70)	3 (30)	3 (30)	7 (70)	6 (60)	4 (40)	8 (80)	2 (20)	10
T _{any} N _{any} M ₁	6 (7)	6 (40)	7 (58)	5 (42)	9 (75)	3 (25)	7 (58)	5 (42)	1 (8)	11 (92)	3 (25)	9 (75)	9 (75)	3 (25)	12
	p < 0.001		p = 0.952		p = 0.949		p = 0.743		p = 0.428		p = 0.252		p = 0.804		97
G1 (Ki67 ≤2)	80 (84)	3 (17)	50 (60)	33 (40)	61 (73)	22 (27)	56 (67)	27 (33)	17 (20)	66 (80)	32 (39)	51 (61)	60 (72)	23 (28)	83
G2 (Ki67 = 3–20)	15 (16)	15 (83)	20 (67)	10 (33)	25 (83)	5 (17)	21 (70)	9 (30)	6 (20)	24 (80)	13 (43)	17 (57)	22 (73)	8 (27)	30
G3 (Ki67 >20)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	00
	p < 0.001		p = 0.347		p = 0.204		p = 0.495		p = 0.592		p = 0.402		p = 0.558		113
Nonfunctioning	96 (96)	17 (89)	72 (64)	41 (36)	86 (76)	27 (24)	76 (67)	37 (33)	24 (21)	89 (79)	47 (42)	66 (58)	83 (73)	30 (27)	113
Functioning	4 (4)	2 (11)	3 (50)	3 (50)	4 (67)	2 (33)	4 (67)	2 (33)	0 (0)	6 (100)	1 (17)	5 (83)	5 (83)	1 (17)	6
	p = 0.244		p = 0.392		p = 0.453		p = 0.643		p = 0.250		p = 0.221		p = 0.506		

The numbers in brackets represent percentages. The results of SSTR4 are not shown as no immunoreactivity was detected in any of the studied tumors. PAD = Pathological diagnosis.
^a Results obtained by the SS-800 SSTR2A antibody provided by Gramsch. ^b Results obtained by the UMB-1 SSTR2A antibody provided by Epitomics.

stroma in the majority of cases with variable extent. Typical examples are depicted in figure 2.

In LCNECs, SSTR expression was occasionally observed at the minority of cells, concerning exclusively SSTR1 and SSTR2A and the intensity was weaker than that observed in LCs. Regarding SCLCs, none of the studied tumors expressed any of the SSTRs. Overall, immunoreactivity for DR2 was absent in all PDECs studied. PDECs had a Ki67 LI higher than 20% as expected.

Co-expression of DR2 with SSTR2A was observed in 75 cases (63%) for the SS-800 and 64 (54%) for the UMB-1 antibody (mean: 59%). The relevant scores for SSTR1, SSTR3 and SSTR5 (Gramsch antibodies), were 67 (56%), 22 (19%) and 44 (37%), respectively. No significant difference was observed in receptor co-expression between TCs and ACs.

Furthermore, the possible association between the expression of SSTRs and DR2 with tumor aggressiveness was assessed. Initially, the tumors were stratified according to their size: 96 tumors were ≤3 cm, whereas 23 tumors were >3 cm. Moreover, patients were grouped according to their disease extent. In 75 patients, the tumor was confined to lung tissue (T_{any}N₀M₀), in 10, there was lymph node involvement (T_{any}N_{1–3}M₀), and in 12, distant metastases were present (T_{any}N_{any}M₁). In 9 patients, data regarding disease extent were not available. No significant difference was found in the distribution of SSTR 1–5 and DR2 compared to any of these parameters. Similar results were obtained when comparing the receptor expression between functioning and nonfunctioning tumors (table 2).

Finally, the tumors were classified according to the Ki67 LI based on the grading criteria proposed by ENETS for gastroenteropancreatic NETs [36]. Of the studied tumors, 83 (73%) were G1 (Ki67 LI ≤2%) and 30 (27%) were G2 (Ki67 LI 3–20%). None of the well-differentiated tumors proved to be G3 (Ki67 LI >20%), whereas in 6 tumors immunostaining for Ki67 was not performed due to insufficient material. The proportion of G2 tumors was significantly higher among ACs compared to TCs (83 vs. 16%, p < 0.001); however, no significant difference was found regarding the distribution of any of the SSTRs or DR2 between G1 and G2 tumors (table 2). In the above comparisons, the expression of SSTR2A was studied separately by using either the SS-800 antibody or the UMB-1 antibody; however the results were similar regardless of the antibody used.

The receptor expression was also evaluated in the 17 metastatic lesions (exclusively Gramsch antibodies available) with the following findings: SSTR1: 82%, SSTR2A:

Table 3. Contingency table summarizing the expression scores of SSTRs 1–5 and DR2 among metastatic lesions in comparison to their corresponding primary tumors

Primary Tumors	Metastases											
	SSTR1		SSTR2A		SSTR3		SSTR4		SSTR5		DR2	
	+	–	+	–	+	–	+	–	+	–	+	–
Positive expression	11	3	14	0	3	3	0	0	8	2	10	2
No expression	0	3	2	1	1	10	0	17	0	7	5	0
Agreement	82% – $k = 0.57$		88% – $k = 0.45$		76% – $k = 0.44$		100%		88% – $k = 0.76$		59% – $k = -0.20$	

SSTR2A immunostaining available only with the Gramsch SS-800 antibody in all the metastases. Positive and negative expression at the metastatic lesions is denoted by the signs + and –, respectively.

82%, SSTR3: 35%, SSTR4: 0%, SSTR5: 59% and DR2: 71%. Concordance between metastatic lesions and the corresponding primary tumors was found for SSTR1 in 14 cases (82%), for SSTR2A in 15 (88%), for SSTR3 in 13 (76%), for SSTR5 in 15 (88%) and for DR2 in 10 (59%) cases (table 3). No significant difference was observed when lymph node and distant metastases were evaluated separately.

Comparison to SRS Data

The presence of concordance between SRS and IHC findings was assessed for SSTR2A, SSTR3 and SSTR5 in 30 tumors (18 primaries with their corresponding metastases) (table 4). This proved to be satisfying only for SSTR2A when assessed by the SS-800 antibody (90% concordance – $k = 0.62$, $p < 0.001$). The concordance rates for SSTR3 and SSTR5 were 37% ($k = -0.22$) and 50% ($k = 0.18$), respectively. The SRS uptake reported according to the KS score correlated positively to the IHC score proposed by Volante et al. [35] for SSTR2A (r_s 0.586, $p = 0.02$); thus, tumors with membranous SSTR2A expression exhibited higher uptake. In particular, 6/10 tumors with clear tumor uptake ($KS \geq 2$) showed membranous SSTR2A immunostaining (S2 and S3), while the remaining 4 showed a cytoplasmic immunostaining pattern (S1). Moreover, all 10 tumors with faint tumor uptake showed cytoplasmic immunostaining (S1). Finally, 3/6 tumors with no tracer uptake were negative for SSTR2A, whereas the remaining 3 had positive SSTR2A immunostaining. The relevant results for SSTR2A were inferior after using the UMB-1 antibody (71% concordance – $k = 0.38$), and the correlation with KS was less strong (r_s 0.492, $p = 0.15$).

Detection of SSTR2A and DR2 Gene Transcripts in LCs

In a subset of 20 LCs, gene transcripts of somatostatin and DRs were analyzed using semiquantitative RT-PCR. Satisfactory mRNA was extracted from 11 cases, and GAPDH (control gene) was amplifiable in 8 cases. Following normalization to GAPDH, a proportion of LCs showed variable amplification of gene transcripts of SSTR1, SSTR2A, SSTR3, SSTR5 and DR2 indicating increased transcription of the genes (fig. 3). The relative incidence of the receptors' mRNA expression was 88, 88, 12.5, 63 and 75%, respectively. A concordance with IHC results for SSTR1, SSTR2A, SSTR3, SSTR5 and DR2 was observed in 63, 88 (75% for UMB-1), 88, 50 and 88%, respectively. All samples tested by RT-PCR were negative for amplification of the SSTR4 gene transcript (table 5).

Discussion

In the present study, we evaluated the expression of SSTRs 1–5 and DR2 in a large series of LCs and in 20 lung PDECs. A relatively high incidence of SSTR2A (72%) and SSTR1 (63%) expression was observed, and to a lesser extent of SSTR5 (40%) and SSTR3 (20%), whereas SSTR4 was not expressed in any of the specimens studied. These findings are in line with the results reported in previous studies evaluating IHC, autoradiography, in situ hybridization and RT-PCR findings in LCs, which have shown a relative expression of SSTR1 in 60–72%, of SSTR2A in 49–96%, of SSTR3 in 4–40%, of SSTR5 in 14–60% and no SSTR4 expression [15, 18–21]. Recently, Tsuta et al. [22] have studied immunohistochemically the differential expression of SSTRs 1–5 in 68 LCs (57 TCs and 11 ACs; us-

Table 4. Patients with available SSTR imaging data

S/N	Material	PAD	SRS	KS	SSTR2A				SSTR3	SSTR5
					SS-800	SS-800 score ^a	UMB-1	UMB-1 score ^a		
1a	PRIM	AC	+	2	+	1	NA	NA	–	–
1b	LNM	AC	+	2	+	1	NA	NA	–	–
2	PRIM	TC	+	3	+	2	+	3	+	+
3	PRIM	TC	–	0	–	0	–	0	–	–
4	PRIM	TC	–	0	–	0	–	0	–	–
5	PRIM	TC	+	1	+	1	–	0	–	–
6	PRIM	TC	+	1	+	1	+	1	–	–
7a	PRIM	AC	+	1	+	1	+	3	–	–
7b	HEP	AC	+	3	+	3	+	3	–	+
8a	PRIM	TC	+	1	+	1	–	0	–	+
8b	HEP	TC	+	2	+	1	–	0	–	+
8c	ADR	TC	+	2	+	1	–	0	–	+
9a	PRIM	TC	+	1	+	1	–	0	–	–
9b	HEP	TC	+	4	+	2	+	2	–	–
9c	LNM	TC	+	1	+	1	+	2	–	–
9d	LNM	TC	–	0	+	3	+	3	+	+
10	PRIM	TC	+	3	+	3	+	2	–	–
11	PRIM	TC	–	0	–	0	–	0	–	–
12	PRIM	TC	+	3	+	3	+	3	+	+
13a	PRIM	TC	–	0	+	1	–	0	–	–
13b	PRIM	TC	–	0	+	1	–	0	+	–
14	PRIM	TC	+	1	+	1	+	2	–	+
15a	PRIM	AC	+	PET+ ^b	+	3	+	3	+	+
15b	HEP	AC	+	PET+	+	3	+	3	+	+
16a	PRIM	TC	+	PET+	+	1	+	2	+	–
16b	LNM	TC	+	PET+	+	1	+	3	–	–
17a	PRIM	TC	+	1	+	1	–	0	–	–
17b	LNM	TC	+	1	+	1	–	0	+	–
18a	LNM	TC	+	1	+	1	+	2	–	–
18b	LNM	TC	+	2	+	3	+	3	+	+

Correlation with immunohistochemical findings. Specimens with the same serial number (S/N) were obtained from the same patient. PAD = Pathological diagnosis; PRIM = primary tumor; LNM = lymph node metastasis; HEP = liver metastasis; ADR = adrenal metastasis.

^aSSTR2A scoring according to the score proposed by Volante et al. [35]. ^b⁶⁸Ga-DOTATOC positron emission tomography positive tumors with SUV max 17.

ing the antibodies provided by Gramsch) and the corresponding results were 83, 96, 47, 4 and 0% for SSTRs 1–5, respectively; however, they applied a different scoring system. Regarding DR2, the expression frequency has been reported to range between 75 and 100% [31, 32]. We have demonstrated a similarly high incidence (74%) of DR2 expression and have additionally documented the co-expression of DR2 with different SSTRs in more than half of the investigated tumors.

No difference was observed regarding SSTR expression between TCs and ACs, whereas the expression of DR2 was more avid in ACs. These findings are not completely in line with the current notion that receptor expression seems to decrease with increasing tumoral dedifferentiation. By contrast, the findings in PDECs complied with this statement, since the expression of all SSTRs and DR2 was negligible in these tumors [19]. These results may favor the opinion of several authors that LCs and lung PDECs form two separate groups of tumors with different

Table 5. RT-PCR amplification results and the corresponding IHC receptor expression as demonstrated by means of IHC shown as positive (+)/negative (–) values as well as agreement scores between the two methods

	RT-PCR Amplification											
	SSTR1		SSTR2A (UMB-1) ^a		SSTR3		SSTR4		SSTR5		DR2	
	+	–	+	–	+	–	+	–	+	–	+	–
IHC expression												
Positive	4	0	6 (5)	0 (0)	0	0	0	0	1	0	5	0
None	3	1	1 (2)	1 (1)	1	7	0	8	4	3	1	2
Agreement	63%		88% (75%)		88% ^b		100% ^b		50%		88%	
	(k = 0.25)		(k = 0.60)						(k = 0.16)		(k = 0.72)	

^a The results in brackets refer to the UMB-1 antibody.

^b k was not computed because SSTR3-IHC and SSTR4-IHC is a constant.

Fig. 3. Detection of SSTRs 1–5 and DR2 by semiquantitative RT-PCR in LCs. The bars represent the level of amplification of the receptor gene products after normalization to GADPH levels. The

symbols + or – above the bars stand for positive or negative immunohistochemical expression, respectively. A group of 8 cases is shown.

properties and variable behavior, rather than comprising a continuum with TCs and SCLCs representing the two extremes and ACs with LCNECs lying in the middle.

The immunoreactivity of SSTRs and DR2 was predominantly cytoplasmic, apart from SSTR2A that was mainly localized on the cell membrane. The clinical significance of cytoplasmic immunostaining was further studied by comparing the expression of SSTR2A by using two different antibodies, one polyclonal (SS-800) and one monoclonal (UMB-1). The relative expression frequencies were comparable (76 vs. 67%), and UMB-1 was associated with a predominantly membranous pattern with less background immunostaining. However, the results obtained by SS-800 correlated better with those obtained by RT-PCR and SRS. In this case, membrane expression of SSTR2A was related to higher SRS uptake ($KS \geq 2$); nevertheless, uptake was also observed among tumors with cytoplasmic SSTR2A expression, though this was less strong ($KS 1$). The presence of receptors in tumors with cytoplasmic staining was confirmed by semiquantitative RT-PCR, and a possible explanation for this is that receptor protein may accumulate in the cytoplasm shortly after its biosynthesis or after internalization as part of receptor metabolism [37, 38]. This issue may also be of importance regarding DR2 and particularly the way we evaluate its co-expression with SSTR subtypes. The internalization of DR2 may prevent receptor dimerization, with SSTRs located on the membrane, which is essential for the effects of chimeric compounds to take place [28].

Among SSTRs, only SSTR2A expression agreed substantially with the SRS findings, a result that was predictable due to the pharmacokinetic properties of the applied tracer (DTPA-octreotide). Moreover, it is of interest that half of the tumors negative to SRS exhibited various expressions of SSTRs immunohistochemically, suggesting that IHC could be an additional and potentially more sensitive predictive marker compared to SRS before applying therapies based on SAs.

An inadvertent finding was the expression of SSTR3 at the tumoral and nontumoral fibrovascular stroma. Previous studies have demonstrated the expression of SSTR2A and SSTR5 in fibrovascular stroma of nontumoral tissues (endothelial and immune cells) [39]; however, the expression of SSTR3 is a relative novel finding. This pattern is mainly encountered in endothelial cells of rapidly proliferating vessels and it might be correlated to neoangiogenesis [40].

The substantial concordance between immunohistochemical and RT-PCR findings, regarding SSTR2A, SSTR3, SSTR4 and DR2, suggests that IHC detects reli-

ably at least these receptors in LCs. In contrast, concerning SSTR1 and SSTR5, the results were not that obvious as RT-PCR demonstrated receptor expression in more tumors compared to IHC. However, this finding could be explained by post-transcription events that may not allow the expression of the genes at the protein level. Besides, RT-PCR has been reported elsewhere to be a more sensitive detecting method than IHC [19].

The assessment of receptor expression in metastatic lesions in comparison to their 'antecedent' primary tumor showed in general fair to good concordance rates regarding SSTR1, SSTR2A and SSTR3 ($k = 0.57, 0.45$ and 0.44 , respectively) and excellent concordance regarding SSTR5 ($k = 0.77$). In contrast, agreement was poor regarding DR2 ($k = -0.2$). According to these results, the SSTR profile of a metastatic lesion may reflect sufficiently the corresponding profile of the primary tumor, whereas this seems not to be the case for DR2.

Currently the standard treatment of LCs is surgery, while there are not guidelines available for medical treatment. In previous studies, SAs have offered efficient symptomatic relief but an effect on tumor growth was found only in a minority of patients [41]. However, more recent studies have demonstrated the ability of SAs to control the growth of well-differentiated small-intestine NETs [42]. In addition, a beneficial antitumoral effect of novel multivalent SAs that can bind to several SSTRs has been shown in LC cell lines [10]. In the present study, SSTR2A, the major target of the currently used SAs, was undetectable in 25–33% of the studied tumors, irrespective of the antibody used (SS-800 or UMB-1). However, 50–75% of these tumors expressed at least one SSTR among SSTR1, SSTR3 and SSTR5, offering the rationale for a therapeutic trial with such multivalent analogues in poor responders to currently used SAs such as octreotide/lanreotide.

The expression of DRs in NETs may also be of therapeutic significance as the DA cabergoline has been shown to exert some effect in pancreatic NETs [43]. Furthermore, synergistic action of SAs and cabergoline has been shown in a patient with refractory Cushing's syndrome due to ectopic adrenocorticotrophin secretion by a LC [27]. Receptor heterodimerization between SSTRs and DRs has been observed [28], and novel chimeric molecules that couple both SSTRs and DR2 – such as BIM23A387 that targets SSTR2A and DR2 and BIM23A760 that targets SSTR2A, SSTR5 and DR2 – have been developed [44, 45]. These agents show at least in vitro more potent antiproliferative effects than each component alone or in combination; thus, demonstrating that tumors co-express DR2 with different SSTRs may help to

identify patients suitable for treatment rather than applying such expensive therapies to the whole cohort. However, a randomized study may be necessary to address this issue.

In summary, this study provides a full mapping of the five SSTR subtypes and assesses their co-expression with DR2 in LCs. Apart from SSTR2A, a high relative incidence of SSTR1 and to a lesser extent of SSTR5 and SSTR3 was observed. This finding and the co-expression of SSTRs and DR2 in a significant number of these tumors could provide a rationale for the possible treatment with agents that target these receptors.

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