Olfactory receptor 51E1 as a novel target for diagnosis in somatostatin receptor-negative lung carcinoids

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Abstract

Somatostatin receptors (SSTRs) may be used in lung carcinoids (LCs) for diagnosis and therapy, although additional targets are clearly warranted. This study aimed to investigate whether olfactory receptor 51E1 (OR51E1) may be a potential target for LCs. OR51E1 coding sequence was analyzed in LC cell lines, NCI-H727 and NCI-H720. OR51E1 transcript expression was investigated in LC cell lines and frozen specimens by quantitative real-time PCR.

OR51E1, SSTR2, SSTR3, and SSTR5 expression was evaluated by immunohistochemistry on paraffin-embedded sections of 73 typical carcinoids (TCs), 14 atypical carcinoids (ACs), and 11 regional/distant metastases and compared with OctreoScan data. Immunohistochemistry results were rendered semiquantitatively on a scale from 0 to 3, taking into account the cellular compartmentalization (membrane vs cytoplasm) and the percentage of tumor cells (<50 vs >50%). Our results showed that WT OR51E1 transcript was expressed in both LC cell lines. OR51E1 mRNA was expressed in 9 out of 12 TCs and 7 out of 9 ACs ($P=NS$).

Immunohistochemically, OR51E1, SSTR2, SSTR3, and SSTR5 were detected in 85, 71, 25, and 39% of TCs and in 86, 79, 43, and 36% of ACs respectively. OR51E1 immunohistochemical scores were higher or equal than those of SSTRs' in 79% of TCs and 86% of ACs. Furthermore, in the LC cases where all SSTR subtypes were lacking, membrane OR51E1 expression was detected in 10 out of 17 TCs and 1 out of 2 ACs. Moreover, higher OR51E1 immunohistochemical scores were detected in 5 out of 6 OctreoScan-negative LC lesions. Therefore, the high expression of OR51E1 in LCs makes it a potential novel diagnostic target in SSTR-negative tumors.

Key Words

- ▶ olfactory receptor 51E1
- ▶ lung carcinoids
- ▶ novel target for diagnosis
- ▶ somatostatin receptors
- ▶ OctreoScan

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Introduction

Neuroendocrine neoplasms of the respiratory tract are divided into well-differentiated tumors, i.e. lung carcinoids (LCs), and poorly differentiated neuroendocrine

carcinomas, i.e. large cell neuroendocrine carcinomas and small-cell lung carcinomas (SCLCs). In turn, LCs are subdivided into typical carcinoids (TCs) and atypical

carcinoids (ACs), the former being low-grade malignant tumors with less than two mitoses per 2 mm², absence of necrosis, and good prognosis ($\approx 88\%$, 5-year survival) and the latter being intermediate-grade malignant tumors with up to ten mitoses per 2 mm² and/or presence of punctate necrosis and poorer prognosis ($\approx 50\%$, 5-year survival) (Beasley *et al.* 2004). Differences in metastatic potential to regional lymph nodes, liver, and bone account for major differences in behavior of TCs and ACs (Beasley *et al.* 2004), with radical surgery offering the best chance to survive these tumors (Filosso *et al.* 2002a).

Because susceptibility to chemotherapy agents of metastatic LCs may be reduced due to low response rates and serious side effects, multimodality strategies have been developed (Gustafsson *et al.* 2008). Indeed, somatostatin receptors (SSTRs) can be expressed in LCs (Righi *et al.* 2010) and may be used for both their diagnosis and therapy (Pelosi *et al.* 2006). In particular, SSTR scintigraphy using ¹¹¹In-DTPA⁰-octreotide (OctreoScan) has been applied to diagnose LCs targeting mainly SSTR2, with an additional affinity to SSTR3 and SSTR5 (Papotti *et al.* 2001). Somatostatin analog (SSA) biotherapy has been shown mainly to control symptoms and improve long-term survival of LC patients, especially those with metastatic ACs (Filosso *et al.* 2002b). Moreover, peptide receptor radionuclide therapy with radiolabeled SSAs, such as [¹⁷⁷Lu-DOTA⁰,Tyr³]-octreotate (van Essen *et al.* 2007a), represents an alternative and valuable treatment in neuroendocrine tumor (NET) patients (Kwekkeboom *et al.* 2011), to cause tumor response or stabilization (van Essen *et al.* 2007a). However, NET patients with negative or low-level uptake in SSTR scintigraphy are not optimal candidates for using SSAs as diagnostic or therapeutic tools (van Essen *et al.* 2007b). Therefore, the development of novel tumor targets in LCs is clinically warranted to improve diagnosis and therapy.

The olfactory receptor (OR) genes belong to the largest superfamily in the human genome (Olender *et al.* 2008). ORs comprise a subclass of G-protein-coupled receptors predominantly expressed in the olfactory sensory neurons where they play a pivotal role in the specific recognition of diverse stimuli (Ache & Young 2005). One of these genes, termed *OR51E1* (family 51, subfamily E, member 1), is poorly understood due to the orphan status of its physiological ligand. However, odorants such as 3-methyl-valeric acid, 4-methyl-valeric acid (Fujita *et al.* 2007), nonanoic acid, and butyl butyryl lactate (Saito *et al.* 2009) have shown high affinity to human *OR51E1*. *OR51E1* mRNA expression

has been detected in normal brain (Vanti *et al.* 2003) and prostate tissue (Weigle *et al.* 2004). Furthermore, cancer cells, such as prostate carcinoma cells (Weigle *et al.* 2004, Fuessel *et al.* 2006, Wang *et al.* 2006, Weng *et al.* 2006) and small intestine neuroendocrine carcinoma (SI-NEC) cells (Leja *et al.* 2009, Cui *et al.* 2013), have been shown to express high levels of *OR51E1*. The pathophysiological role of *OR51E1* in tumorigenesis and tumor progression has not been elucidated. However, *OR51E1* has been suggested as a novel diagnostic marker complementary to other traditional markers in prostate cancers (Wang *et al.* 2006) and can significantly distinguish tumors from normal tissue and benign hyperplasia in the prostate (Weng *et al.* 2006). Although *OR51E1* does not associate with the aggressiveness of prostate cancer, lower *OR51E1* expression correlates with earlier recurrence (Weng *et al.* 2006).

The aim of this study was to investigate the *OR51E1* coding sequence, the relevant protein and mRNA expression in LCs, normal lung tissue, various types of lung cancer, and established LC cell lines. The type of subcellular immunohistochemical localization of *OR51E1* in LCs was compared with that of SSTR2, SSTR3, and SSTR5. The SSTR2, SSTR3, and SSTR5 protein expression was also correlated with the *in vivo* OctreoScan images in LC patients to further consolidate the possible diagnostic value of the SSTR immunohistochemical expression. Our results indicated that *OR51E1* is likely to represent a novel target for diagnosis in LCs, alternative to SSTRs.

Subjects and methods

Cell culture

LNCAp cells, from a prostate carcinoma, NCI-H727 cells, from a TC, and NCI-H720 cells, from an AC, were purchased from American Type Culture Collection (ATCC, University Boulevard, Manassas, VA, USA) and cultured as recommended by the catalog. KRJ-1 and CNDT2.5, both SI-NEC cells, were gifts from Prof. I M Modlin (Yale University, New Haven, CT, USA) and Prof. L M Ellis (Anderson Cancer Centre, Houston, TX, USA) respectively and were cultured as described previously (Modlin *et al.* 2006, van Buren *et al.* 2007). Cell culture media components comprised DMEM-F12, F12, RPMI-1640, sodium pyruvate, MEM vitamin, HEPES, and NEAA, all purchased from Life Technologies; PEST and L-glutamine from the National Veterinary Institute (SVA, Uppsala, Sweden); and FBS from Sigma.

Patients and tumors

Frozen specimens were obtained from 31 patients with lung neuroendocrine neoplasms, including 12 TCs (three males and nine females), nine ACs (three males and six females), and ten SCLCs (five men and five females). Moreover, five lung adenocarcinomas and five squamous cell carcinomas, matched for age and sex, were used as a lung tumor-related control group. The tumor cell population was on average about 80% in each specimen. In addition, normal surrounding lung tissue was available from eight of the aforementioned LC patients (four TCs and four ACs).

Furthermore, the study included surgical specimens from 86 primary tumors (72 TCs and 14 ACs), ten lymph node metastases from TCs, and one liver metastasis from an AC. In addition, another laser-treated primary TC was also included. The specimens derived from a total of 80 patients, including 66 patients with TCs, of which six had multiple tumors and 14 patients with ACs. The median age at operation was 52 years for TC patients (22 males and 44 females) and 51 years for AC patients (five males and nine females). The average primary tumor size was 2.0 ± 2.5 cm (mean $\pm$ 2 s.d.) in TCs and 2.6 ± 2.9 cm in ACs. Two patients with TCs emerged under the spectrum of multiple endocrine neoplasia type 1 syndrome. Four patients had clinical syndromes attributed to inappropriate hormone secretion from the tumor, two with Cushing's syndrome due to ectopic adrenocorticotrophin secretion, one with typical, and one with AC syndrome. Four TC patients had been treated before operation, one received paraptatin and etoposide, two temozolomide, and one interferon α . Of these, one patient concomitantly received SSA for control of the carcinoid syndrome.

In 14 cases, i.e. 12 TC patients and two AC patients, after injecting 175–220 MBq ^{111}In -DTPA-octreotide, OctreoScan was performed preoperatively and 19 focal lesions comprising 14 primary tumors, four lymph node metastases, and one liver metastasis were graded according to the method of Kwekkeboom *et al.* (2005).

Controls

Additional specimens from normal pancreas obtained from a patient operated on for a non-functioning pancreatic NET and specimens from a patient operated on for a prostate carcinoma (Gleason grade 5), were used as positive controls for the SSTRs and OR51E1 respectively. The specificity of all reactions was checked by replacing the primary antibody with a non-related rabbit immunoglobulin (rabbit IgG, cat no. I-1000) purchased from

Vector Labs (Burlingame, CA, USA) at a comparable dilution. Positive and negative controls were used as appropriate. In occasional cases, i.e. four primary TCs, one lymph node metastasis deriving from a TC case, and one liver metastasis from an AC, neutralization test was conducted by a 24-h incubation of the primary antibody with the relevant antigen, i.e. 100 μg antigen/ml diluted antibody solution, before application to the sections. The antigens for SSTRs were obtained from Gramsch Laboratories (Schwabhausen, Germany): SSTR2A (SS-801), SSTR3 (SS-851), and SSTR5 (SS-891) and from Epitomics, Inc. (Burlingame, CA, USA): SSTR2 (P-3582-1); the antigen for OR51E1 (P1854) was from LSBio (Seattle, WA, USA).

RNA extraction and cDNA synthesis

All the included cell lines were cultured for fewer than ten passages before RNA extraction. Total RNA from LNCaP, NCIH-727, NCIH-720, CNDT2.5, and KRJ1 cells was isolated using a PARIS Kit from Life Technologies, whereas total RNA from the normal fibroblast 1064SK cells was a gift from Prof. M Essand's group (IGP, Rudbeck Laboratory, Uppsala University, Uppsala, Sweden). Furthermore, about 3–5 μg total RNA from each frozen specimen was isolated using the RNeasy Mini Kit from Qiagen. The RNA 6000 Nano Kit and the Agilent 2100 Bioanalyzer from Agilent Technologies (Santa Clara, CA, USA) were used to verify the quality and quantity of all isolated total RNAs. The cDNAs was prepared using the cDNA synthesis kit from Bio-Rad for the cell lines and the MuLV reverse transcriptase from Life Technologies for the frozen specimens.

OR51E1 transcript sequencing

The OR51E1 coding sequence was cloned from the cDNA of NCI-H727 and NCI-H720 cells. PCR was performed using forward primer, 5'-TCA GCT TCT TCA TGA TGG TGG-3' and reverse primer, 5'-CAC TGA CAC CTA GGG CTC TGA-3'. The TOPO TA Cloning Kit from Life Technologies was used for cloning and the OR51E1 coding sequence was analyzed at the Rudbeck Laboratory, Uppsala Genome Centre (Uppsala, Sweden; http://www.genpat.uu.se/facilities/genome_center/). Sequences were then aligned with GenBank published data.

Quantitative real-time PCR

Gene-specific PCR products were measured in the cell line cDNAs by Agilent Stratagene Mx3005P real-time PCR system with Brilliant II SYBR Green QPCR Master Mix

from Agilent Technologies and in the tissue cDNAs by iCycler IQ real-time detection system with iQ SYBR Green Supermix from Bio-Rad. Each PCR contained about 10 ng cDNA when dealing with either cell lines or frozen tumor tissues, in the latter case, by cutting an adequate number of 10 μ m-thick sections comprising at least 75% tumor cellularity. Relative expression of OR51E1 was evaluated after normalization against β -actin expression, using the same primers and method as described previously (Leja *et al.* 2009), where relative fold changes were calculated for each tumor patient in comparison with the median value for normal tissue in all the patients.

Immunohistochemistry and scoring system

The tissue specimens were fixed in 10% buffered neutral formalin for 18–20 h at room temperature, followed by routine processing to paraffin wax. Approximately 4 μ m thick sections were cut and attached to positively charged glass slides (Superfrost⁺) from Menzel Gläser (Braunschweig, Germany). The sections were microwave treated for 2 $\times$ 5 min at 700 W using 50 mM Tris buffer saline, pH 8.0, as retrieval solution and immunostained using a polymer detection system (Dako Cytomation, EnVision+ System-HRP, K4010) from Dako (Glostrup, Denmark). Diaminobenzidine was used as chromogen. The antibodies were diluted in Dako's antibody diluent (S3022). The SSTR antibodies were polyclonal rabbit from Gramsch Laboratories: SSTR2A (SS-800, 1:5000), SSTR3 (SS-850, 1:5000), and SSTR5 (SS-890, 1:5000) and monoclonal rabbit anti-SSTR2A antibody, UMB-1, from Epitomics, Inc. (1:200). OR51E1 polyclonal rabbit antibody (A1854, 1:500) was purchased from LSBio. The sections were incubated with the primary antibodies overnight at room temperature. Meyer's hematoxylin from Histolab (Gothenburg, Sweden) was used for nuclear counterstain. Immunostaining was evaluated, in consecutive immunostained sections, under a light microscope (Axioskop 40) from Carl Zeiss (Oberkochen, Germany) and the percentage of immunoreactive (IR) tumor cells was estimated at a magnification of $\times$ 400 using a grid in one of the oculars. At least four randomly selected areas were examined; in small lesions, the entire neoplastic tissue was investigated. Images were obtained at the same magnification using an Axiophot light microscope from Carl Zeiss and AxioVision Rel. 4.5 Software.

Immunohistochemical results for the relevant antibodies were rendered semiquantitatively on a scale from 0 to 3, taking into account the cellular compartmentalization (membrane vs cytoplasm) and the percentage of tumor cells (<50 vs >50%), which slightly modifies a

previously released scoring system for SSTR2 (Volante *et al.* 2007). Briefly, pure cytoplasmic immunoreactivity in <50% tumor cells and/or membrane immunoreactivity (partial or circumferential) in <10% tumor cells was scored as 0 (S0) (this modification was done in agreement with autoradiography results (Korner *et al.* 2012)); pure cytoplasmic immunoreactivity in >50% tumor cells as 1 (S1); incomplete membranous immunoreactivity (<50% of cell circumference) in 10–50% tumor cells, irrespective of the presence of cytoplasmic immunoreactivity, as 2 (S2); and circumferential membranous immunoreactivity ($\geq$ 50% of cell circumference) in >50% tumor cells, irrespective of the presence of cytoplasmic immunoreactivity, as 3 (S3). In addition, we selected the highest immunohistochemical score for SSTR2 from the results obtained using the two different SSTR2 antibodies.

Statistical analysis

Surgical specimens from 72 primary TCs, 14 primary ACs, and 11 regional/distant metastases were analyzed in all the statistical analyses, whereas another laser-treated primary TC was evaluated for OctreoScan comparison only. In detail, the Wilcoxon-matched pairs signed rank test was used to compare immunohistochemical score for OR51E1 and the highest score among SSTR2, SSTR3, and SSTR5 for each specimen. The Mann–Whitney *U* test was used to evaluate the statistical difference in OR51E1 mRNA expression between TCs and ACs and to assess the difference in protein expression of each receptor between TCs and ACs, between different stages of tumors, and between smaller (<3 cm) and larger ($\geq$ 3 cm) primary LCs, according to the protocol of the American Joint Committee on Cancer classification. The Kruskal–Wallis test, with Dunn's multiple comparison *post-hoc* analysis, was used to evaluate the difference among SSTR2, SSTR3, and SSTR5 immunohistochemical scores. The Fisher's exact test was used to assess the concordance between the OctreoScan grades and the SSTR2 immunohistochemical scores of the corresponding tumors. The Spearman's correlation analysis was used to access the correlation of immunohistochemical scores analyzed by three different investigators. All the analyses were two-tailed and performed using GraphPad Prism 5 from GraphPad Software (La Jolla, CA, USA). All *P* values <0.05 were considered significant.

Ethics statement

The entire research protocol was reviewed and approved by the research ethics boards at Uppsala University

Hospital (Sweden; ref. no. Dnr 2011/051), and the internal revision board of the European Institute of Oncology (Milan, Italy). All the patients included in the study signed a document giving informed consent.

Results

WT *OR51E1* transcript expression in established human LC cell lines

The sequence analysis showed the absence of any mutation in the *OR51E1* coding sequence from both the TC NCI-H727 cells and the AC NCI-H720 cells. The sequence was identical to the original WT *OR51E1*, which was published on GenBank (accession number: NP_689643.2). Quantitative real-time PCR (QRT-PCR) analysis revealed similar *OR51E1* transcript expression between NCI-H727 and NCI-H720 cells. In contrast, no *OR51E1* expression was detected in the other investigated established NET cells, i.e. CNDT2.5 and KRJ1 cells (Fig. 1).

OR51E1 transcript expression in both typical and atypical LCs

QRT-PCR analysis revealed *OR51E1* transcript expression in 9 out of 12 TCs and 7 out of 9 ACs ($P=NS$ (not significant) between TCs and ACs), of which 5 out of 12 TCs and 2 out of 9 ACs showed high expression (Table 1).

Figure 1

OR51E1 transcript expression in established human neuroendocrine tumor cells. *OR51E1* expression was evaluated by QRT-PCR and normalized to β -actin (set as 1). s.d.s of triplicate samples are shown. NCI-H727, typical lung carcinoid; NCI-H720, atypical lung carcinoid; KRJ-1 and CNDT2.5, small intestine neuroendocrine carcinomas. LNCaP cells, from a prostate carcinoma, were used as a positive control and 1064SK cells, from normal skin fibroblast, were used as a negative control.

Table 1 Relative *OR51E1* transcript expression in different types of lung cancer cells

	–	+	++	+++
TCs (n=12)	3/12	3/12	1/12	5/12
ACs (n=9)	2/9	3/9	2/9	2/9
SCLCs (n=10)	3/10	6/10	1/10	0/10
ADCs (n=5)	3/5	2/5	0/5	0/5
SCCs (n=5)	1/5	3/5	1/5	0/5

TC, typical carcinoid; AC, atypical carcinoid; SCLC, small-cell lung carcinoma; ADC, adenocarcinoma; SCC, squamous cell carcinoma; –, negative expression; +, low expression; ++, intermediate expression; +++, high expression.

Predominant *OR51E1* expression was detected in both the TC and AC tumor cells compared with the normal surrounding bronchopulmonary cells (Table 2). Furthermore, *OR51E1* transcript expression was detected in 7/10 SCLCs, 2/5 lung adenocarcinomas, and 4/5 lung squamous cell carcinomas, whereas high *OR51E1* expression was not observed in these carcinomas (Table 1).

Immunohistochemistry and statistical analysis

Lung carcinoids Based on our proposed scoring system (S0–S3), *OR51E1* and *SSTR2*, *SSTR3*, and *SSTR5* expressions on all the LC tumor sections were evaluated by three different investigators (T C, L G, and A V T). The immunohistochemical scores for each of the four receptors showed high correlation (Spearman's correlation coefficient $\rho > 0.9$) between any two different investigators of the three. Thus, this scoring system is highly reproducible for the present investigation. Furthermore, we used the median scores obtained by the three investigators, for each receptor and on each tumor specimen, during our subsequent statistical analyses.

Table 2 Relative *OR51E1* transcript expression in TC and AC cells in comparison with normal bronchopulmonary cells from the same patients

TCs (n=4)	T1	+++	N1	–
	T2	+++	N2	–
	T3	+++	N3	–
	T4	–	N4	+
ACs (n=4)	T1	+++	N1	–
	T2	+	N2	–
	T3	–	N3	–
	T4	–	N4	–

TC, typical carcinoid; AC, atypical carcinoid; T, carcinoid tumor cells; N, normal bronchopulmonary cells; –, negative expression; +, low expression; ++, intermediate expression; +++, high expression.

Figure 2

OR51E1 immunostaining in lung carcinoids. Immunostaining patterns in the neoplastic cells of typical carcinoids are illustrated as non-immunoreactive, score 0 (A); pure cytoplasmic, score 1 (B); incomplete membranous in <50% of tumor cells, score 2 (C); and circumferential membranous in >50% of tumor cells, score 3 (D). Representative immunostaining in lymph node metastasis, score 3, from a typical carcinoid (E), and in liver metastasis, score 2, from an atypical carcinoid (F), are also shown. Neutralized immunostaining in the same cell clusters is shown in each inset. Bar = 50 μ m.

OR51E1 immunostaining according to the scoring system is depicted in Fig. 2. OR51E1 protein was highly expressed mainly in the tumor cell membrane of both primary TCs and ACs and of the regional/distant metastases, with an inverse correlation being detected between OR51E1 and SSTRs. Indeed, the S0 group included most SSTR-negative tumors relative to OR51E1 cases, whereas the S3 group harbored the highest distribution of OR51E1-positive tumors relative to SSTR cases, with intermediate distributions being found in S1 and S2 groups (Fig. 3). In total, OR51E1, SSTR2, SSTR3, and SSTR5 were detected (corresponding to immunohistochemical scores S1, S2, and S3) in 85, 71, 25, and 39% of primary TCs and in 86, 79, 43, and 36% of primary ACs respectively whereas 79% (57/72) of primary TCs had higher or equal scores for OR51E1 compared with highest score for SSTR2, SSTR3, and SSTR5 ($P=0.0083$) (Table 3). This percentage for the

TC lymph node metastases was 70% (7/10). Although not statistically significant ($P=0.1246$), this percentage for the primary ACs was 86% (12/14), whereas the AC liver metastasis was categorized as S2 for OR51E1 and S3 for SSTR2. Furthermore, no significant difference in OR51E1 scores, or in each SSTR score, was detected between primary TCs and ACs, between different stages of disease, i.e. primaries and metastases, or between smaller (<3 cm) and larger (≥ 3 cm) primary LC tumors. Thus, OR51E1 and each SSTR were distributed uniformly across the entire spectrum of LCs.

Figure 3

Summary of SSTR2, SSTR3, SSTR5, and OR51E1 immunohistochemical scores in primary typical (A) and atypical carcinoids (B). X-axis shows score 0 (S0) to S3, whereas y-axis shows the percentage of analyzed tumors (indicated above the columns), which were immunostained for each receptor and classified in each scoring category. The number of tumors that correlates with each percentage, in 72 primary typical carcinoids, is S0: 21, 54, 44, 11 vs S1: 9, 14, 23, 1 vs S2: 17, 4, 5, 28 vs S3: 25, 0, 0, 32 for SSTR2, SSTR3, SSTR5, and OR51E1 respectively and, in 14 primary atypical carcinoids, is S0: 3, 8, 9, 2 vs S1: 2, 5, 5, 0 vs S2: 6, 1, 0, 3 vs S3: 3, 0, 0, 9 for SSTR2, SSTR3, SSTR5, and OR51E1 respectively. All the numerical values as described above represent calculations based on the median immunohistochemical scores obtained by the three investigators, whereas the ranges of the scores are indicated by the error bars for each column.

Table 3 Comparison of OR51E1 immunohistochemical score with the highest SSTR2, SSTR3, and SSTR5 immunohistochemical score in lung carcinoids

Type	Primary or metastasis	OR51E1 > SSTRs	OR51E1 = SSTRs	OR51E1 < SSTRs	P value
TC	Primary	27/72 (37%)	30/72 (42%)	15/72 (21%)	0.0083
TC	Lymph node metastasis	4/10 (40%)	3/10 (30%)	3/10 (30%)	NS
AC	Primary	8/14 (57%)	4/14 (29%)	2/14 (14%)	NS
AC	Liver metastasis	0/1	1/1	0/1	NA

TC, typical carcinoid; AC, atypical carcinoid; NS, not significant; NA, not applicable.

Moreover, the analysis of 22 SSTR-non-IR (S0) lesions revealed that membrane localization (S2 or S3) of OR51E1 was detected in the tumor cells in 10 out of 17 primary TCs, 2 out of 3 TC lymph node metastases, and 1 out of 2 primary ACs. Furthermore, 7 out of 9 primary TCs, 2 out of 3 TC lymph node metastases, and 2 out of 2 primary ACs showed membrane OR51E1 immunohistochemical pattern (S2 or S3), where SSTR2 was predominantly localized in the cytoplasm (S1) (Fig. 4).

In addition, we observed higher SSTR2 expression compared with SSTR3 and SSTR5 ($P < 0.05$) in both types of LCs, whereas no difference was detected between SSTR3 and SSTR5. Between the immunohistochemical scores obtained using the two SSTR2 antibodies, 16 out of 72 primary TCs and 5 out of 14 primary ACs showed higher scores when the clone UMB-1 SSTR2 antibody from Epitomics, Inc. was used, whereas 8 out of 72 TCs and 2 out of 14 ACs showed higher scores using the SSTR2 antibody from Gramsch Laboratories.

Controls Distinct cells with membrane immunoreactivity were identified in the pancreatic islets and in prostate carcinoma cells for SSTRs and OR51E1 respectively. No immunoreactivity was seen after replacing the relevant primary antibodies with the non-related rabbit immunoglobulin or after the neutralization test.

OR51E1 protein is expressed in LCs with negative OctreoScan

A comparison between functional imaging and immunohistochemical results was performed on 12 primary TCs, two primary ACs, and five metastases, i.e. four lymph node metastases from TCs and one liver metastasis from an AC, for which OctreoScan data were available. In total, 4 out of 6 OctreoScan-negative lesions were immunohistochemically scored 0 (S0) for SSTR2, 4 out of 6 OctreoScan grade 1 lesions were scored 1 (S1), and 7 out of 7 OctreoScan grades 2–4 lesions were scored 2–3 (S2–S3). Thus, the concordance rate was 79% (15 out of 19 lesions)

between the OctreoScan grades and the corresponding SSTR2 immunohistochemical scores ($P < 0.001$).

At variance with SSTRs, OR51E1 immunoreactivity was uniformly distributed across the tumors for which OctreoScan data were available regardless of uptake levels (Table 4). Indeed, OR51E1 scores (S2–S3) were higher than SSTR2, SSTR3, and SSTR5 (S0–S1) in 5 out of 6 OctreoScan-negative TC lesions (five primary tumors and one lymph node metastasis). OR51E1 showed high scores (S2–S3) in 5 out of 6 OctreoScan grade 1 lesions and in 7 out of 7 OctreoScan grade 2–4 lesions, where moreover, 3 out of 6 grade 1 lesions and 2 out of 7 grade 2–4 lesions presented higher scores for OR51E1 than all the SSTR subtypes ($P = 0.042$ for OR51E1 scores vs highest SSTR scores, in all the 19 LC lesions).

Figure 4

Representative immunostaining with higher OR51E1 score compared with SSTR2, SSTR3, and SSTR5 in a primary typical carcinoid. OR51E1 shows membrane pattern staining in the tumor cells (score 3). The inset highlights OR51E1 membranous staining with a higher magnification ($\times 800$), of the tumor cells marked by the red frame. SSTR2 (photo obtained from immunostaining by using anti-SSTR2, clone UMB1) and SSTR3 exhibit mainly cytoplasmic staining in these tumor cells (score 1), for which neutralized immunostaining are shown in the insets. The tumor is non-immunoreactive for SSTR5 (score 0). Bar = 50 μ m.

Table 4 SSTR2, SSTR3, SSTR5, and OR51E1 immunohistochemical scores in lung carcinoids with OctreoScan data

Patient	Type	Primary or metastasis	OctreoScan	SSTR2	SSTR3	SSTR5	OR51E1
1	TC	Primary	No uptake	S0	S0	S0	S3
2	TC	Primary	No uptake	S0	S0	S0	S2
3	TC	Primary	No uptake	S0	S0	S0	S0
4	TC	Primary	No uptake	S1	S0	S0	S2
	TC	Primary	No uptake	S1	S1	S0	S3
5	TC	Lymph node metastasis	No uptake	S0	S0	S0	S2
	TC	Lymph node metastasis	Grade 1	S1	S2	S2	S2
6	TC	Primary	Grade 1	S2	S0	S0	S0
7	TC	Primary	Grade 1	S2	S0	S1	S2
	TC	Lymph node metastasis	Grade 1	S1	S1	S1	S3
8	TC	Primary	Grade 1	S1	S0	S0	S2
9	TC	Primary	Grade 1	S1	S0	S0	S3
10	TC	Primary	Grade 3	S3	S1	S1	S3
11	TC	Primary	Grade 3	S2	S0	S1	S3
	TC	Lymph node metastasis	Grade 3	S2	S0	S1	S3
12	TC	Primary	Grade 4	S2	S0	S0	S2
13	AC	Primary	Grade 2	S3	S0	S0	S2
	AC	Liver metastasis	Grade 3	S3	S0	S1	S2
14	AC	Primary	Grade 3	S3	S1	S1	S3

TC, typical carcinoid; AC, atypical carcinoid; S0, score 0, non-immunoreactive specimens, pure cytoplasmic immunoreactivity in <50% of tumor cells and membrane immunoreactivity (partial or circumferential) in <10% of tumor cells; S1, score 1, pure cytoplasmic immunoreactivity in >50% of tumor cells; S2, score 2, incomplete membranous immunoreactivity (<50% of cell circumference) in 10–50% of tumor cells, irrespective of the presence of cytoplasmic immunoreactivity; S3, score 3, circumferential membranous immunoreactivity (≥50% of cell circumference) in >50% of tumor cells, irrespective of the presence of cytoplasmic immunoreactivity. Octreoscan data were graded according to the method described by Kwekkeboom *et al.* 2005.

Discussion

SSTRs may be variably expressed in typical and atypical LCs (Papotti *et al.* 2001, Pelosi *et al.* 2006, Righi *et al.* 2010, Tsuta *et al.* 2012) and may be used in both their diagnosis and treatment. However, novel molecular targets are needed to improve the clinical management of these patients. Here, we indicated that OR51E1, a G-protein coupled receptor, showed a higher or similar expression compared with SSTR2, SSTR3, and SSTR5 in 79% (57 out of 72) of primary TCs, 86% (12 out of 14) of primary ACs, and in 73% (8 out of 11) LC metastases. High membrane OR51E1 expression was found in 10 out of 17 primary TCs and 1 out of 2 primary ACs that were non-IR for SSTRs and moreover in 5 out of 6 OctreoScan-negative TC lesions. Furthermore, OR51E1 showed more extensive membrane localization than SSTRs in 7 out of 9 primary TCs and 2 out of 2 primary ACs exhibiting cytoplasmic immunostaining for SSTRs and moreover in 3 out of 6 OctreoScan grade 1 TC lesions. These findings indicate that OR51E1 may play an important role as a potential novel target in the diagnosis of both TCs and ACs, irrespective of their SSTR expression.

The predominant OR51E1 expression by both immunohistochemistry and QRT-PCR in tumors when compared with normal lung tissue, its distribution across

the spectrum of LCs, and the lack of mutations in the relevant gene supports the potential of OR51E1 as a stable molecule in these tumors to target for diagnostic purposes. For the OR51E1 receptor, the relevance of membrane localization to diagnosis of NETs is heralded by the use of other G-protein-coupled receptors, e.g. SSTRs, as mainly membrane rather than cytoplasmic immunoreactivity should be considered for a standardized and reliable clinical report (Volante *et al.* 2007). In our study, OR51E1 has shown more extensive membrane localization compared with SSTR2, SSTR3, and SSTR5, which are targeted by most of the clinically available SSAs. These findings indicated that OR51E1 might display a higher uptake in LC cells, compared with the SSA-based diagnostics, after developing appropriate procedures of immuno-positron emission tomography (PET; Knowles & Wu 2012) by means of OR51E1 monoclonal antibodies with high receptor binding affinity. Indeed, OR51E1 has shown an advantage over SSTRs as it has prevalence across the entire spectrum of LCs, even in OctreoScan-negative and/or SSTR non-IR LCs, independent of tumor size. This heightens the opportunity to use OR51E1 as a complement to SSTRs in diagnosing these tumors.

In conclusion, our study indicates that OR51E1 may be a potential novel target for LCs, especially when dealing with SSTR-negative tumor patients. Tumor diagnosis

based on OR51E1 immuno-PET analysis is an interesting possibility for better management of these challenging tumor patients irrespective of their SSTR status, although further studies are required to test these possibilities.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

V G, T C, G P, and A V T conceived and designed the experiments. T C, L G, G P, and A V T performed the experiments. V G, T C, L G, G P, and A V T analyzed the data. V G, K Ö, G P, and A V T contributed reagents/materials and analysis tools. V G, T C, L G, G P, and A V T wrote the paper.

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