CLINICAL STUDY

Olfactory receptor 51E1 protein as a potential novel tissue biomarker for small intestine neuroendocrine carcinomas

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Abstract

Objective: Late diagnosis hinders proper management of small intestine neuroendocrine carcinoma (SI-NEC) patients. The olfactory receptor, family 51, subfamily E, member 1 (OR51E1) has been reported as a potential novel SI-NEC marker, without protein expression recognition. Thus, we further studied whether the encoded protein may be a novel SI-NEC clinical biomarker.

Design: OR51E1 coding sequence was cloned using total RNA from SI-NEC patient specimens. Quantitative real-time PCR analysis explored OR51E1 expression in laser capture microdissected SI-NEC cells and adjacent microenvironment cells. Moreover, immunohistochemistry investigated OR51E1 protein expression on operation and biopsy material from primary SI-NECs, mesentery, and liver metastases from 70 patients. Furthermore, double immunofluorescence studies explored the potential co-localization of the vesicular monoamine transporter 1 (SLC18A1, generally referred to as VMAT1) and OR51E1 in the neoplastic cells and in the intestinal mucosa adjacent to the tumor.

Results: OR51E1 coding sequence analysis showed absence of mutation in SI-NEC patients at different stages of disease. OR51E1 expression was higher in microdissected SI-NEC cells than in the adjacent microenvironment cells. Furthermore, both membranous and cytoplasmic OR51E1 immunostaining patterns were detected in both primary SI-NECs and metastases. Briefly, 18/43 primary tumors, 7/28 mesentery metastases, and 6/18 liver metastases were 'positive' for OR51E1 in more than 50% of the tumor cells. In addition, co-localization studies showed that OR51E1 was expressed in > 50% of the VMAT1 immunoreactive tumor cells and of the enterochromaffin cells in the intestinal mucosa adjacent to the tumor.

Conclusion: OR51E1 protein is a potential novel clinical tissue biomarker for SI-NECs. Moreover, we suggest its potential therapeutic molecular target development using solid tumor radioimmunotherapy.

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Introduction

Neuroendocrine carcinomas (NECs) are rare malignancies mainly characterized by an indolent clinical course and a delayed diagnosis. Small intestine NECs (SI-NECs) originate from enterochromaffin (EC) cells in the intestinal mucosa. In general, metastatic disease is present at the time of diagnosis and mainly involves the mesenteric lymph nodes and the liver. The major medical treatment for metastatic SI-NECs comprises biotherapy, using somatostatin analogs (SSAs), interferon α (1), and lately everolimus (2). SSAs relieve the patients' symptoms and also have a major role in controlling tumor growth (3). In addition, treatment with radiolabelled SSAs is a well-established treatment

in the management of patients with inoperable SI-NECs (4). Therefore, hormonal receptors, such as somatostatin receptors, became pivotal tissue markers to assess the therapeutic effectiveness of SSAs (5). However, some SI-NEC patients are not responsive to SSA treatment (6). Thus, there is an unmet need of finding novel markers to accelerate and improve the clinical management of SI-NEC patients.

The *OR51E1* gene encodes the olfactory receptor, family 51, subfamily E, member 1, and the OR genes belong to the largest receptor superfamily in humans (7). The ORs are mainly expressed in the cilia of the olfactory sensory neurons and play an essential role in the specific recognition of diverse stimuli (8). They are G-protein-coupled receptors, which have been pivotal

for drug development and therapeutic approaches against a variety of diseases including cancer (9). As neurons and neuroendocrine cells are phenotypically similar (10), scientists started exploring whether the ORs were also expressed in the EC cells of the normal intestinal mucosa and whether they may be involved in the serotonin release mechanism. Kidd *et al.* (11) revealed that different olfactants mediate the luminal serotonin release from normal and neoplastic human EC cells. Furthermore, Braun *et al.* (12) detected the expression of four ORs in the microdissected EC cells of the intestinal mucosa and investigated the downstream signaling pathway.

Leja *et al.* have previously identified six differentially expressed novel genes, including the *OR51E1* gene in SI-NECs. In spite of these findings, OR51E1-encoded protein had not been detected at that time (13). Although the OR51E1 physiological ligand is unknown, Fujita *et al.* (14) recognized that molecules such as 3-methyl-valeric acid and 4-methyl-valeric acid are able to increase cAMP concentration via a chimeric OR51E1 signaling. OR51E1 mRNA expression was first detected in brain tissues (15) and soon after in normal prostate cells (16). Moreover, OR51E1 has been described as a novel mRNA tissue marker for prostate cancers (16, 17, 18, 19). However, OR51E1 protein expression has never been investigated in either prostate tissue or any tumor material. Thus, in this study, the principal aim was investigating whether OR51E1 protein can be detected in SI-NECs and developed as a new clinical tissue biomarker for these tumors.

Materials and methods

Ethics statement

Patient samples were included in the study after a written consent statement was obtained from each individual. The study was performed in accordance with the regional ethics committee at Uppsala University, Sweden (ref. no. Dnr 2011/426).

Tissue specimens

Snap-frozen tissue specimens ($n=9$; primary tumors ($n=3$), mesentery metastases ($n=3$), and liver metastases ($n=3$)) from patients with histopathologically confirmed SI-NEC diagnosis were included in the laser capture microdissection (LCM), RNA extraction, and cDNA synthesis studies. Furthermore, formalin-fixed paraffin-embedded tissue specimens ($n=90$) from patients ($n=71$) with SI-NEC at different stages of disease (primary tumors ($n=43$), mesentery metastases ($n=28$), and liver metastases ($n=18$)) were included in the immunohistochemical examinations. The median age at operation of SI-NEC patients (men ($n=30$) and women ($n=41$)) was 62 years (range 33–82 years). From all the primary tumor cases and liver metastases,

sufficient adjacent normal tissue was also available. In addition, one tissue specimen from a prostate carcinoma patient (Gleason grade 5) was also included. Approximately 4 μm -thick sections were cut and attached to positively charged glass slides (Superfrost⁺, Menzel Gläser, Braunschweig, Germany). Patients' clinicopathological data are summarized in Tables 1 and 2.

Laser capture microdissection

Snap-frozen tissue specimens were sliced into $\sim 10 \mu\text{m}$ sections using a microtome cryostat (Leica, Solms, Germany) and adhered to polyethylene naphthalate membrane frame slides (Life Technologies). Tumor cells and cells of adjacent tumor microenvironment, from each section, were extracted using the Arcturus^{XT} microdissection system (Life Technologies) according to the manufacturer's instructions.

RNA extraction and cDNA synthesis

Total RNA from snap-frozen SI-NEC specimens was isolated using mirVana miRNA isolation kit (Life Technologies), whereas total RNA from LCM tumor cells and LCM cells of the adjacent tumor microenvironment were prepared using the RNeasy Micro Kit (Life Technologies). About 1 μg of total RNA from frozen blocks and 3 ng of total RNA from LCM cells were reverse transcribed into cDNA using cDNA synthesis kit (Bio-Rad), after DNase I treatment with DNA-free kit (Life Technologies).

OR51E1 transcript sequencing

OR51E1 coding sequence was amplified using 10 ng cDNA from total RNA of six SI-NEC specimens; forward primer 5'-TCA GCT TCT TCA TGA TGG TGG-3' and reverse primer 5'-CAC TGA CAC CTA GGG CTC TGA-3'. The PCR products were cloned using the TOPO TA Cloning kit (Life Technologies) and sequenced at the Uppsala Genome Center, Rudbeck Laboratories, Uppsala, Sweden (http://www.genpat.uu.se/facilities/genome_center/). Sequences were then aligned with GenBank published data.

Quantitative real-time PCR

About 400 pg cDNA from LCM cells, as described earlier, were used as template of each reaction. Relative OR51E1 expression was measured using previously described primers (13). The reactions were run using Brilliant II SYBR Green QPCR Master Mix and Agilent Stratagene Mx3005P real-time PCR system (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. The analysis was then normalized with β -actin expression by the $2^{-\Delta\Delta C_t}$ method (20).

Table 1 Frozen tissue specimens used for sequencing and microdissection.

Sex/age	Type	Tissue	Treatment	Application
M/80	P	Ileocecal	Untreated	LCM, Seq
F/63	P	Ileum	Untreated	LCM, Seq
F/63	P	Ileum	Untreated	LCM, Seq
F/51	M	Mesentery	SSA, IFN	LCM
M/56	M	Mesentery	SSA, IFN	LCM, Seq
F/77	M	Mesentery	SSA, IFN	LCM, Seq
F/40	L	Liver	SSA, IFN	LCM
F/76	L	Liver	SSA, IFN	LCM, Seq
F/65	L	Liver	SSA, IFN	LCM

P, primary tumor; M, mesentery metastasis; L, liver metastasis; age, age at operation; SSA, somatostatin analog; IFN α , interferon α ; LCM, laser capture microdissection; Seq, sequencing.

Immunohistochemistry

The sections were microwave treated for 2 $\times$ 5 min at 700 W and 1 $\times$ 15 min at 380 W using 10 mM citrate buffer, pH 6, as retrieval solution, before immunostaining. The sections were immunostained using a polymer detection system (DakoCytomation, EnVision + System-HRP, cat. no. K4011, Dako, Glostrup, Denmark), then incubated at 4 °C overnight with the rabbit polyclonal anti-human OR51E1 antibody (cat. no. A1854, LSBio, Seattle, WA, USA), and diluted 1:500 in Dako antibody diluent (cat. no. S3022, Dako). Diaminobenzidine was used as chromogen. Nuclear counterstain was performed using Meyer's hematoxylin (Histolab Product, Gothenburg, Sweden). The sections were evaluated under Axiophot light microscope (Carl Zeiss, Oberkochen, Germany) and images were obtained using AxioVision Rel. 4.5 Software (Carl Zeiss). The percentage of the immunoreactive (IR) cells was estimated by a light microscope at a magnification of $\times$ 400 using a square grid in one of the oculars. Four randomly selected areas were at least examined on tissue specimens, whereas the entire neoplastic tissue was examined for the smaller lesions. Two methods were used for the immunohistochemical classification of the tumors. In the first method (quantitative), the tumors were considered as 'positive' if OR51E1 immunoreactivity appeared in the majority (> 50%) of the tumor cells, independently of the cytoplasmic or membranous immunostaining pattern. In the second method (semi-quantitative), the subcellular localization of the receptor was taken into consideration as follows: pure cytoplasmic immunoreactivity, membranous immunoreactivity in < 50% of the cell circumference (or partial membranous immunoreactivity), and membranous immunoreactivity in over 50% of the cell circumference (or complete circumferential immunoreactivity). In addition, OR51E1 'positive' cells in the intestinal mucosa adjacent to the tumor were counted. The density of IR cells, expressed as the number of IR cells per one high power field (HPF) mucosa area, was

calculated using a square grid in one of the oculars at a magnification of $\times$ 400. Fifteen HPF/specimen in parallel to the muscularis mucosa were evaluated from sufficient adjacent mucosa on six primary SI-NEC cases.

Prostate carcinoma specimen expressing OR51E1 was used to optimize the method and as a 'positive' control in subsequent experiments. In addition, 'negative' control studies were performed to validate the sensitivity and specificity of OR51E1 antibody as follows: i) replacement of the primary antibody, on all the tissue specimens, with nonimmune rabbit IgG (cat. no. I-1000, Vector Labs, Burlingame, CA, USA) at the same dilution and in the same diluent, as described earlier; and ii) neutralization test, using 24-h incubation of the primary antibody with the respective blocking peptide (cat. no. P1854, LSBio) before application on the specimens conducted on primary SI-NECs ($n=3$), mesentery metastasis ($n=1$), and liver metastasis ($n=1$).

Co-localization studies were performed in selected sections. The specimens were microwave treated as mentioned earlier and blocked for 30 min with normal donkey serum (cat. no. 017-000-121, Jackson ImmunoResearch, West Grove, PA, USA) at room temperature. Then the specimens were incubated at 4 °C overnight with a mixture of two primary antibodies that included 1:400 diluted goat polyclonal anti-human vesicular monoamine transporter 1 (VMAT1, also known as SLC18A1, solute carrier family 18 member1) antibody (C-19, cat. no. sc-7718, Santa Cruz Biotech, Santa Cruz, CA, USA) and 1:100 diluted OR51E1 antibody (cat. no. A1854, LSBio). Then the sections were incubated for 30 min at room temperature with a mixture of fluorescence-labeled secondary antibodies, which included fluorescein (FITC)-donkey anti-goat (cat. no. 705-095-147, Jackson ImmunoResearch) and rhodamine (TRITC)-donkey anti-rabbit (cat. no. 711-025-152, Jackson ImmunoResearch), both diluted 1:100 in PBS. Vectashield (cat. no. H-1000, Vector Labs) was used for the mounting of the specimens. The immunostaining was evaluated under AxioPlan2 imaging microscope (Carl Zeiss). Images were acquired with a $\times$ 630 magnification and analyzed with AxioVision Rel. 4.8 Software (Carl Zeiss). In addition, the intestinal mucosa adjacent to the tumor was examined in five primary SI-NEC cases with a view to investigate a possible co-expression between VMAT1- and OR51E1-IR cells. Photos were taken from a total of 35–50 randomly selected HPFs per case. The double-immunostained specimens were photographed with the 63 $\times$ plan-apochromat objective, and the number of cells that co-expressed VMAT1 and the OR51E1 was counted in the merged pictures. Similarly, the possible co-expression of the transporter and the receptor in question in the tumor cells was evaluated in 15–20 HPF/specimen at a magnification of $\times$ 630, on five primary SI-NECs, two mesentery metastases, and two liver metastases cases.

Table 2 Summary of OR51E1 immunostaining in the tumor cells of SI-NECs.

Type	Treatment	OR51E1 immunoreactivity				Total (%)
		C (%)	PM (%)	CM (%)	M (%)	
Muc ^a	NA	NA	NA	NA	NA	NA
P ^{a,b}	Untreated	58	2	0	2	60
P	Untreated	2	1	0	1	3
P	Untreated	17	3	0	3	20
P	Untreated	72	10	3	13	85
P	Untreated	5	0	0	0	5
P	Untreated	4.8	0.2	0	0.2	5
P	Untreated	9.5	0.5	0	0.5	10
P	Untreated	1.8	0.2	0	0.2	2
P ^{a,b}	Untreated	54	5	1	6	60
P	Untreated	4.5	0.5	0	0.5	5
P	Untreated	1.8	0.2	0	0.2	2
P	Untreated	3.8	1	0.2	1.2	5
P	Untreated	20	8	2	10	30
P	Untreated	2.9	0.1	0	0.1	3
P	Untreated	1.8	0.2	0	0.2	2
P	Untreated	9.49	0.5	0.01	0.51	10
P	Untreated	4	1	0	1	5
P	SSA	3	1	1	2	5
P ^{a,b}	SSA	25	15	15	30	55
P ^{a,b}	SSA	73	2	0	2	75
P	SSA	84	5	1	6	90
P	SSA	3	0	0	0	3
P	SSA	29	5	1	6	35
P	SSA	60	5	5	10	70
P	IFN	10	5	60	65	75
P	IFN	29.9	0.1	0	0.1	30
P	IFN	9	1	0	1	10
P ^{a,b}	IFN	27.5	2	0.5	2.5	30
P	IFN	44	10	1	11	55
P	SSA, IFN	20	20	20	40	60
P	SSA, IFN	4.8	0.2	0	0.2	5
P	SSA, IFN	9.5	0.5	0	0.5	10
P ^b	SSA, IFN	25	25	30	55	80
P	SSA, IFN	30	35	20	55	85
P	SSA, IFN	40	15	5	20	60
P	SSA, IFN	61	8	1	9	70
P	SSA, IFN	38	15	2	17	55
P	SSA, IFN	55	20	5	25	80
P	SSA, IFN	9.9	0.1	0	0.1	10
P	SSA, IFN	8	2	0	2	10
P	SSA, IFN	55	10	5	15	70
P	SSA, IFN	23	5	2	7	30
P	SSA, IFN	53	2	0	2	55
M	Untreated	48	5	2	7	55
M	Untreated	0.95	0.05	0	0.05	1
M	Untreated	0.95	0.05	0	0.05	1
M	Untreated	29.45	0.5	0.05	0.55	30
M	Untreated	9.5	0.5	0	0.5	10
M	Untreated	3	2	0	2	5
M	Untreated	2	0	0	0	2
M ^a	Untreated	55	10	5	15	70
M	Untreated	1	0	0	0	1
M	Untreated	10	10	0	10	20
M	Untreated	33	20	2	22	55
M	Untreated	0.9	0.1	0	0.1	1
M	Untreated	3	2	0	2	5
M	Untreated	24	1	0	1	25
M	Untreated	0.9	0.1	0	0.1	1
M	Untreated	0	0	0	0	0
M	SSA	20	0	0	0	20
M	IFN	0.9	0.1	0	0.1	1
M	IFN	27.5	2	0.5	2.5	30
M	SSA, IFN	34	20	1	21	55
M	SSA, IFN	15	20	55	75	90
M	SSA, IFN	2	0	0	0	2
M	SSA, IFN	0.95	0.05	0	0.05	1

Table 2 Continued

Type	Treatment	OR51E1 immunoreactivity				Total (%)
		C (%)	PM (%)	CM (%)	M (%)	
M	SSA, IFN	69	10	1	11	80
M	SSA, IFN	32	8	0	8	40
M	SSA, IFN	0.9	0.1	0	0.1	1
M	SSA, IFN	4	1	0	1	5
M ^a	SSA, IFN	65	10	5	15	80
L	Untreated	4	1	0	1	5
L	Untreated	1	1	1	2	3
L	Untreated	8.8	1	0.2	1.2	10
L ^a	Untreated	51	2	2	4	55
L	Untreated	8.5	1	0.5	1.5	10
L	Untreated	0	0	0	0	0
L ^a	Untreated	35	15	20	35	70
L	Untreated	52	2	1	3	55
L	Untreated	25	5	0	5	30
L	SSA	3.5	1	0.5	1.5	5
L	SSA	0	0	0	0	0
L	SSA	0	0	0	0	0
L	IFN	40	10	10	20	60
L	IFN	45	5	5	10	55
L	SSA, IFN	25	30	5	35	60
L	SSA, IFN	5	5	0	5	10
L	SSA, IFN	1	1	0	0	1
L	SSA, IFN	0	0	0	0	0

Muc, ileal mucosa adjacent to the primary tumors; P, primary tumor; M, mesentery metastasis; L, liver metastasis; NA, not applicable SSA, somatostatin analog; IFN α , interferon α ; C (%), percentage cytoplasmic immunostaining; PM (%), percentage partial membrane immunostaining; CM (%), percentage circumferential membrane immunostaining; M (%), percentage total membrane immunostaining; Total (%), percentage total OR51E1 immunostaining.

^aSections also used for co-localization studies.

^bSections used for counting OR51E1 immunoreactive cells in the intestinal mucosa adjacent to the tumor.

Statistical analysis

The paired Student's *t*-test was used to assess the statistical significance of the *OR51E1* transcript expression between the microdissected tumor cells and the microdissected cells of the adjacent tumor microenvironment, from the same patients. The analyses were two tailed and performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). All *P* values <0.05 were considered significant.

Results

Wild-type *OR51E1* coding sequence from established NEC cells and SI-NEC specimens

The sequence analysis showed the absence of any mutation in the *OR51E1* coding sequence from SI-NEC patient specimens at different stages of disease. Our sequence is identical to the original wild-type *OR51E1*, which was published on GenBank (accession number: NP_689643.2; data not shown).

OR51E1 transcript expression on microdissected SI-NEC cells and microdissected adjacent tumor microenvironment cells

OR51E1 was differentially expressed in the microdissected SI-NEC cells from the nine patients. Significant

lower expression was detected in the microdissected cells of the adjacent tumor microenvironment from all patients (*P* < 0.01).

Immunohistochemistry

About 90% of the prostate carcinoma cells were IR for the *OR51E1* (external 'positive' control) and showed circumferential immunohistochemical pattern. In the intestinal mucosa adjacent to the tumor cells, 0.9

Figure 1 *OR51E1* immunoreactivity in SI-NEC specimens at different stages of disease. Numbers of specimens are clearly shown for different proportions of *OR51E1* 'positive' tumor cells in this figure.

cells/HPF (median; range 0.2–4 cells/HPF) were IR for the OR51E1 (internal 'positive' control). The immunohistochemical pattern detected in the aforementioned cells was either cytoplasmic or membranous.

Immunohistochemistry results for SI-NECs are shown in Table 2. According to the quantitative evaluation of 89 SI-NEC specimens, 31/89 specimens showed over 50% OR51E1 IR tumor cells, specifically 18/43 of the

primary SI-NECs, 7/28 of the mesentery metastases, and 6/18 of the liver metastases (Fig. 1). Cytoplasmic OR51E1 immunostaining pattern was seen in 80% of the IR tumor cells. The strongest signal was clearly detected in the tumor cells facing the fibrovascular stroma. Both partial and complete circumferential OR51E1 membranous immunostaining were detected in 20% of the IR tumor cells at different stages of disease.

Figure 2 OR51E1 Immunohistochemistry on 89 SI-NEC specimens. Representative 'negative' immunostaining (A) and complete cytoplasmic immunostaining (B1) of OR51E1 are illustrated in primary tumor cells, P. The strongest immunoreactivity was detected in the cells (solid arrows) facing the fibrovascular stroma (hollow arrow) (B2). Partial membranous immunostaining (C) and complete circumferential membranous immunostaining (D) are illustrated in primary tumors (C1 and D1) and mesentery metastasis, M (C2 and D2). Perinuclear localization is depicted in a primary tumor specimen (E, arrows). Liver metastases, L, express OR51E1 (F1). No immunoreactive tumor cells were detected after neutralization with the blocking peptide (F2). Bar = 50 μ m.

Perinuclear OR51E1 immunostaining was detected in ~20% of the tumor cells from patients at different stages of SI-NECs. Representative immunostaining patterns of OR51E1 in SI-NECs are illustrated in Fig. 2. No immunoreactivity was detected either after replacement of the antibody by nonimmune serum or after the neutralization test in SI-NECs. Hepatic cell staining in the evaluated liver metastatic specimen persisted in the neutralization test, suggesting the presence of a nonspecific immunostaining in liver.

OR51E1 co-localizes with VMAT1 in the majority of EC cells and neoplastic SI-NEC cells

The co-localization studies revealed that 60% of the EC cells recognized by VMAT1, also known as SLC18A1, express OR51E1 (Fig. 3, upper panel, white arrows), whereas 40% of the EC cells are non-IR for OR51E1 (Fig. 3, lower panel, cyan arrows). In addition, 55% of the mucosa cells are IR for OR51E1 but not IR for VMAT1. In the tissue specimens from patients at different stages of SI-NEC, 64% of the VMAT1-IR tumor cells express OR51E1, whereas 91% of OR51E1-IR tumor cells co-localize with VMAT1 (Supplementary Figure 1, see section on supplementary data given at the end of this article). OR51E1 intracellular distribution pattern was predominant in ~86% of the OR51E1-IR tumor cells. Of these, 14% also displayed membranous immunostaining in addition to cytoplasmic subcellular localization (Fig. 3, white arrows), whereas exclusive membranous immunostaining (Fig. 3, cyan arrows) was detected in about 13% of the OR51E1-IR tumor cells.

Figure 3 VMAT1 and OR51E1 co-localization studies in intestinal mucosa cells lying adjacent to primary SI-NEC. VMAT1 is visualized as green color (FITC), whereas OR51E1 is red (TRITC). Yellow color in the merged images indicates co-localization of the two proteins. The white arrows in the upper panel show one of the OR51E1-IR EC cells (median 60%; range 44–78%; and 93/160 EC cells). The cyan arrows in the lower panel show two of the OR51E1 non-IR EC cells (median 40%; range 22–56%; and 67/160 EC cells). Bar = 20 μ m.

Figure 4 OR51E1 and VMAT1 double immunofluorescence in a primary SI-NEC specimen. VMAT1 is visualized as green color (FITC) and OR51E1 as red (TRITC). Merged images show VMAT1 and OR51E1 co-localization in yellow. Both membranous and cytoplasmic immunostaining patterns (white arrows) are shown in about 14% (median; range 8–24%; and ~2726/26 250 cells) of the OR51E1-IR tumor cells. Exclusive membranous immunostaining (cyan arrows) is shown in about 13% (median; range 9–21%; and ~1508/26 250 cells) of the OR51E1-IR tumor cells. Bar = 20 μ m.

Discussion

It has been previously reported that OR51E1 is differentially expressed in SI-NECs (13). This finding prompted us to study whether OR51E1-encoded protein can be developed as a novel SI-NEC biomarker. Indeed, in this study, the absence of OR51E1 coding sequence mutations on SI-NEC patients' tumor material was confirmed. Furthermore, we showed that OR51E1 RNA is predominantly expressed in the microdissected tumor compartments of all the SI-NEC specimens, at different stages of disease, compared with the surrounding microenvironment (Fig. 4). Leja *et al.* (13) have previously suggested similar results. However, the authors did not use specific microdissection of SI-NEC cells vs the cells of adjacent tumor microenvironment to narrow the cell type.

The immunohistochemical results of OR51E1, on 89 SI-NEC specimens at different stages of disease, revealed predominant OR51E1 protein expression in the tumor cells of the primaries compared with the metastases. It is plausible that in some cases, the metastatic cells lose the ability to produce OR51E1. However, the number of metastases included in this study was too limited to draw certain conclusions. We also detected the strongest OR51E1 positivity in the stroma-facing tumor cell clusters, which may imply the potential role of OR51E1 in the interaction with the surrounding microenvironment (21). About 80% of OR51E1-IR tumor cells showed cytoplasmic immunostaining. Indeed, it is well known that the GPCR cytoplasmic protein portion might be responsible for receptor internalization (22), and to address this concept, it may be necessary to investigate OR51E1 internalization as well. Moreover, OR51E1 was detected in the perinuclear compartment of about 20% of SI-NEC cells. Although this pattern is not common, the ectopically expressed recombinant G-protein-coupled estrogen receptor (GPER) has been found with

perinuclear accumulation shortly after ligand addition (23). Thus, OR51E1 perinuclear immunostaining might reflect an unusual process similar to the GPER one, in which delayed sorting and accumulation of OR51E1 may occur in the perinuclear compartment after endocytosis. Moreover, the OR51E1 protein analysis suggested only OR51E1 nonspecific immunostaining on normal hepatic cells. In addition, we also have an unpublished quantitative real-time PCR analysis of normal human hepatic cells that shows absence of *OR51E1* mRNA expression. These data suggest undetectable levels or absence of OR51E1 in normal liver, a major metastatic organ for SI-NEC patients, which would otherwise have been a major concern if OR51E1 was developed as therapeutic target.

OR51E1 immunostaining was depicted in intestinal cells. However, the density of immunostaining was about 1 cell/×400 HPF examined mucosa. We therefore proceeded with our investigation on the potential co-localization of OR51E1 and VMAT1, also known as SLC18A1, which is a well-established specific marker of EC cells (24) and SI-NEC cells (25). Our findings indicated that the majority (60%) of EC cells lying in the mucosa adjacent to the tumor express OR51E1. Taking into consideration that the mRNA levels in the normal EC cells were about ten times lower than those in the SI-NEC cells from most patients (13), OR51E1 levels may be under the detection threshold of immunofluorescence studies in the non-IR EC cells. An alternative explanation is that two subpopulations of EC cells defined by the presence or lack of OR51E1 receptor expression may exist. Similar results were obtained from the co-localization studies in the tumor specimens. Thus, we suggest that the neoplastic cells expressing VMAT1 and the receptor may derive from the EC cells that show immunoreactivity for OR51E1, whereas the tumor cells uniquely expressing VMAT1 may originate from the non-IR OR51E1 EC cells. Although OR51E1 function should be studied further, the abundant expression of the OR51E1 in a fraction of SI-NEC cases makes it an attractive candidate target in future diagnostics and/or therapeutics.

In conclusion, we have detected OR51E1 protein expression in tissue specimens from SI-NEC patients at different stages of disease. Furthermore, 35% (31 out of 89) of the specimens expressed OR51E1 in more than 50% of tumor cells. These novel findings support OR51E1 as a potential clinical tissue biomarker for a subpopulation of SI-NECs. Indeed, OR51E1 may be developed as a target for novel diagnostics such as immuno-positron-emission tomography (26) and radio-immunotherapy (27) and/or novel potential therapeutics. We also plan to investigate OR51E1 function in both EC cells and SI-NECs in the near future.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-12-0814>.

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

T Cui, A V Tsolakis, K Öberg, and V Giandomenico conceived and designed the experiments. T Cui, A V Tsolakis, S-C Li, and J L Cunningham performed the experiments. T Cui, A V Tsolakis, J L Cunningham, and V Giandomenico analyzed the data. A V Tsolakis, J L Cunningham, K Öberg, and V Giandomenico contributed reagents/materials, analysis tools. A V Tsolakis, T Cui, J L Cunningham, and V Giandomenico wrote the paper.

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