Neuroendocrinology Letters Volume 39 No. 4 2018
ISSN: 0172-780X; ISSN-L: 0172-780X; Electronic/Online ISSN: 2354-4716
Web of Knowledge / Web of Science: Neuroendocrinol Lett
Pub Med / Medline: Neuro Endocrinol Lett

ORIGINAL ARTICLE

Regional differences in somatostatin receptor 2 (SSTR2) immunoreactivity is coupled to level of bowel invasion in small intestinal neuroendocrine tumors

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Submitted: 2018-08-01 Accepted: 2018-10-02 Published online: 2018-10-20

Key words: **SI-NET; SSTR2; immunohistochemistry; invasion**

Neuroendocrinol Lett 2018; **39**(4):305–309 **PMID:** 30531706 NEL390418A07

Abstract

OBJECTIVE: Somatostatin receptor (SSTR) expression constitutes a pivotal cornerstone for accurate radiological detection and medical treatment of small intestinal neuroendocrine tumors (SI-NETs), and the development of somatostatin analogues for these purposes have revolutionized the clinical work-up. Previous assessments of SSTR isoform expression in SI-NETs have found correlations to overall prognosis and treatment response, however these analyses usually report overall tumoral immunoreactivity, and little is reported regarding histo-regional differences in expressional patterns.

METHODS: Thirty-seven primary SI-NETs (WHO grade I, n=32 and WHO grade II, n=5) were collected and assessed for SSTR2 immunohistochemistry. Samples were stratified with regards to histological level of bowel infiltration and spread (mucosal region, muscularis propria region, subserosal region) and each of these tumoral regions was separately scored by SSTR2 staining localization (membrane, cytoplasmic), overall staining intensity and local staining differences within each region.

RESULTS: SSTR2 immunoreactivity was progressively weaker as the tumor cells advanced through the small intestinal layers. This was exemplified by a reduction in the amount of tumor samples with strong SSTR2 expression in the deeper histological levels of the section; 56% of tumors displayed strong SSTR2 expression in the mucosal region, as compared to 29% and 30% of tumors within muscularis propria and subserosal layers, respectively.

CONCLUSIONS: This observation indicates a down-regulation of SSTR2 expression as the tumors progress through the intestinal wall, which might signify underlying biological processes of importance for SI-NET invasion behavior.

INTRODUCTION

Small intestinal neuroendocrine tumors (SI-NETs) are the most commonly encountered neuroendocrine tumors, with an increasing incidence of 1.1 in 100,000 (Pape *et al.* 2012). Genetic and epigenetic studies have identified multiple chromosomal and DNA methylation aberrations in SI-NETs, including the loss of chromosome 18 and 16 and global DNA hypomethylation. (Hashemi *et al.* 2013; Fotouhi *et al.* 2014; Karpathakis *et al.* 2016). Primary SI-NETs are usually small and slow-growing, but metastatic disease is most often already present at the time of diagnosis. Somatostatin analogs (SSAs) have the last decades been the first-line medical treatment to relieve symptoms, but also hinder SI-NET proliferation in some cases (Caplin *et al.* 2014).

Somatostatin receptor 2 (SSTR2) is widely expressed in SI-NET cell lines and tumors and constitutes the main target of natural somatostatin and SSAs for therapy and diagnosis (Oberg *et al.* 2010; Fotouhi *et al.* 2016). Its involvement in the SI-NET SSA therapy is basically due to its inhibitory role on the excess secretion of hormones and substances, which is characteristic of these tumors (Kaltsas *et al.* 2017). SSA therapy enhances the natural autocrine and paracrine somatostatin antisecretory and antiproliferatory effects. By inhibiting adenylyl cyclase and cAMP production it prevents Ca^{++} influx, thereby inhibiting substances' secretion from tumoral cells (Theodoropoulou & Stalla 2013).

Tumoral growth control function has been suggested for the somatostatin-SSTR2 axis, both indirectly and directly. Indirect growth inhibition refers to somatostatin-SSTR2 interactions that lead to lower expression of growth hormone and IGF-1 and inhibition of angiogenesis (Woltering 2003; Murray *et al.* 2004). Upon binding to its ligand, SSTR2 can also induce phosphotyrosine phosphatases SHP-1 and SHP-2 in some cells, leading to dephosphorylation of signal transduction proteins such as ERK1/2, hence direct tumor growth inhibition (Weckbecker *et al.* 2004).

SSTR2 expression in neuroendocrine tumors were previously demonstrated (Brunner *et al.* 2017), however, in this study, we investigated the expression pattern of SSTR2 expression along the direction of SI-NET primary invasion from mucosal to muscularis propria and subserosal region and found that the expression of the receptor is reduced as the tumor develops spatially. The knowledge regarding regional differences in SSTR2 expression might bear implication for the clinical work-up of SI-NET patients, including postoperative tumoral analyses of SSTR2 immunohistochemistry with regards to subsequent imaging and treatment options using SSAs.

MATERIALS AND METHODS

In total, formalin-fixated paraffin-embedded (FFPE) tumor material from 37 primary SI-NETs (n=32 WHO grade 1 and n=5 WHO grade 2) were sectioned, stained and scored for SSTR2 immunoreactivity. A brief clinical presentation of these 37 patients is presented in Supplementary Table 1. Ethical approval was granted by the local ethics committee, and informed consent was available. This study was thus performed in accordance with the ethical standards laid down in an appropriate version of the Declaration of Helsinki.

The SSTR2 staining was performed in an accredited pathology laboratory using a Ventana Benchmark Ultra system (Ventana Medical Systems, Tucson, AZ, USA). Four μm sections from each tissue sample were de-paraffinized using xylen and ethanol. Antigen retrieval was performed using citrate buffer and standardized heating in a microwave oven. Staining was performed using a rabbit monoclonal SSTR2 antibody, (clone UMB1, ab134152, Abcam, Cambridge, UK) at a dilution 1:200. De-identified normal pancreatic tissues were used as a positive control. Staining patterns were assessed by conventional light microscopy by an experienced endocrine pathologist (CCJ).

Samples were stratified with regards to level of bowel infiltration and spread (mucosal region, muscular region, subserosal region) and each of these tumoral regions were scored with regards to SSTR2 staining localization (membrane, cytoplasmic), overall staining intensity (ranked 0–3) and local staining differences within each region (diffuse or partial expression). The immunoreactivity for membranous and cytosolic staining for each region was scored 0 to 3 respectively (0 – absent, 1 – weak, 2 – moderate, 3 – strong) followed by a summarized calculation as the added value for both membranous and cytoplasmic scores, this score ranged from 0–6, in which 0–3 was denoted as "low expression tumors" and 4–6 as "high expression tumors").

Statistical analyses were carried out to assess eventual correlations between clinical parameters and histo-regional SSTR2 immunoreactivity patterns (Mann-Whitney U, Fisher's Exact Test and Kaplan-Meier survival analyses), using SPSS 20.0. *P*-values <0.05 were considered statistically significant.

RESULTS

The staining results of the 37 SI-NET tumors are summarized in Table 1, and examples of different SSTR2 immunohistochemical staining patterns are presented in Figure 1. Tumors were deemed regionally "informative" if tumor cells were present in the corresponding histological layer. Using the proposed algorithm for each individual histological layer, 20 (56%) SI-NETs were classified as exhibiting high SSTR2 expression and 16 (44%) exhibited low SSTR2 expression out of 36 informative cases in the mucosal region. This ratio was statistically different from tumors invading the lamina muscularis propria, in which 10 cases (29%) were classified as exhibiting high expression and 24 cases (71%) displayed low expression out of 34 informative cases (p=0.02). Finally, in the subserosal region, 6 informative

Tab. 1. Regional SSTR2 immunohistochemistry results from the SI-NET cohort.

Tumor	Mucosal M	Mucosal C	Mucosal M+C	Mucosal low or high	Muscularis M	Muscularis C	Muscularis M+C	Muscularis low or high	Subserosal M	Subserosal C	Subserosal M+C	Subserosal low or high
1	2	2	4	High	0	1	1	Low	–	–	–	–
2	3	2	5	High	3	2	5	High	2	1	3	Low
3	2	2	4	High	1	2	3	Low	0	1	1	Low
4	3	3	6	High	2	2	4	High	–	–	–	–
5	0	3	3	Low	0	1	1	Low	–	–	–	–
6	0	2	2	Low	0	1	1	Low	–	–	–	–
7	0	1	1	Low	0	1	1	Low	0	1	1	Low
8	3	3	6	High	0	1	1	Low	–	–	–	–
9	0	3	3	Low	0	3	3	Low	–	–	–	–
10	–	–	–	–	3	2	5	High	3	2	5	High
11	0	3	3	Low	0	2	2	Low	0	2	2	Low
12	0	1	1	Low	0	1	1	Low	0	0	0	Low
13	0	1	1	Low	–	–	–	–	–	–	–	–
14	0	2	2	Low	0	1	1	Low	–	–	–	–
15	0	3	3	Low	0	3	3	Low	–	–	–	–
16	2	2	4	High	0	2	2	Low	0	2	2	Low
17	3	3	6	High	0	2	2	Low	0	1	1	Low
18	2	2	4	High	0	1	1	Low	0	2	2	Low
19	2	2	4	High	2	2	4	High	–	–	–	–
20	3	3	6	High	0	2	2	Low	–	–	–	–
21	0	1	1	Low	0	1	1	Low	0	1	1	Low
22	1	3	4	High	1	3	4	High	1	3	4	High
23	0	2	2	Low	0	2	2	Low	0	2	2	Low
24	3	3	6	High	3	3	6	High	–	–	–	–
25	2	2	4	High	–	–	–	–	–	–	–	–
26	2	2	4	High	0	2	2	Low	2	2	4	High
27	1	2	3	Low	0	2	2	Low	–	–	–	–
28	2	3	5	High	2	3	5	High	2	3	5	High
29	1	2	3	Low	0	1	1	Low	–	1	–	–
30	3	3	6	High	0	1	1	Low	3	3	6	High
31	3	3	6	High	3	3	6	High	–	–	–	–
32	0	3	3	Low	0	3	3	Low	0	3	3	Low
33	0	1	1	Low	0	1	1	Low	1	2	3	Low
34	1	3	4	High	–	–	–	–	–	–	–	–
35	1	1	2	Low	2	2	4	High	2	2	4	High
36	3	3	6	High	2	2	4	High	–	–	–	–
37	3	3	6	High	1	2	3	Low	0	1	1	Low

M – membranous staining, C – cytoplasmic staining; Immunoreactivity levels: 0 – absent, 1 – weak, 2 – moderate, 3 – strong; Summarized M+C scores for each region: 0–3 was denoted as "low expression tumors" and 4–6 as "high expression tumors"

cases (32%) showed high expression and 13 cases (68%) exhibited low expression out of 19 informative cases.

When comparing individual tumors with manifestations of SSTR2 immunoreactivity at different histological layers of the small intestine, 10 out of 20 (50%) "high expression" cases from the mucosal region were classified as demonstrating low expression in the underlying muscularis region, suggesting a reduction in SSTR2

expression as the tumor progresses deeper through the bowel wall. No obvious differences in SSTR2 expression between muscularis and subserosal regions were seen (Table 1).

A number of tumors exhibited a patchy staining pattern with local staining differences within each histological region, a phenomenon that we termed "partial expression" (data not shown). In cases with partial expression, parts of the tumor stained positive for cytoplasmic and/or membranous SSTR2 while other parts of tumor cells within the same region were completely devoid of immunoreactivity. When observed, only the strongest visualized intensity for each case and region was scored to avoid over-complexity of the results. The phenomenon with patchy stainings was seen within the mucosal region in 4/36 (11%) of informative tumors, within the muscularis region in 14/34 (41%) of informative tumors and in the subserosal region in 5/22 (23%) of informative tumors.

A tendency for a worse outcome in patients with tumors exhibiting lower SSTR2 immunoreactivity was seen in our material (Supplementary Figure 1). Moreover, using Fisher's Exact test, a significant correlation between membranous and cytoplasmic immunoreactivity was seen between different histological layers, including mucosal membranous and mucosal cytoplasmic (p=0.02), muscularis membranous and muscularis cytoplasm (p=0.01), mucosal membranous and muscularis membranous (p=0.04), muscularis membranous and subserosal membranous (p=0.004), mucosal cytoplasm and muscularis cytoplasm (p=0.047) stainings respectively.

In addition, we found a significantly increased risk of persistent disease at follow-up in patients with lower SSTR2 expression in the mucosal region, as they exhibited a statistically significant lower expression of SSTR2 (p=0.015) when assessing cytoplasmic staining only (score 0–1 vs. 2–3).

DISCUSSION

Somatostatin and its main receptor, SSTR2, are crucial components in the diagnosis and treatment of SI-NET, lately manifested by the successful implementation of SSAs for hampering tumor progression (Rinke *et al.* 2009; Caplin *et al.* 2014). The constitutional expression of SSTR2 in neuroendocrine tissues and its independent prognostic characteristic in SI-NETs (Brunner *et al.* 2017) suggest a physiological role for somatostatin-SSTR2 axis in the neuroendocrine homeostasis and SI-NET pathogenesis.

In this study we hypothesized that the reduced expression of SSTR2 correlates with SI-NET development and progression. We compared the expression of the protein along the tumors' invasion and progression path, from the small intestine mucosal region to the muscularis propria and subserosal regions. We found indications for a gradual loss of protein expression concomitant with the tumor progression from mucosal to muscularis propria and subserosal region. We believe this could constitute an interesting indication for a physiological role that SSTR2 may play in the maintenance of the differentiated state of the neuroendocrine cells in the mucosal region. The expression of

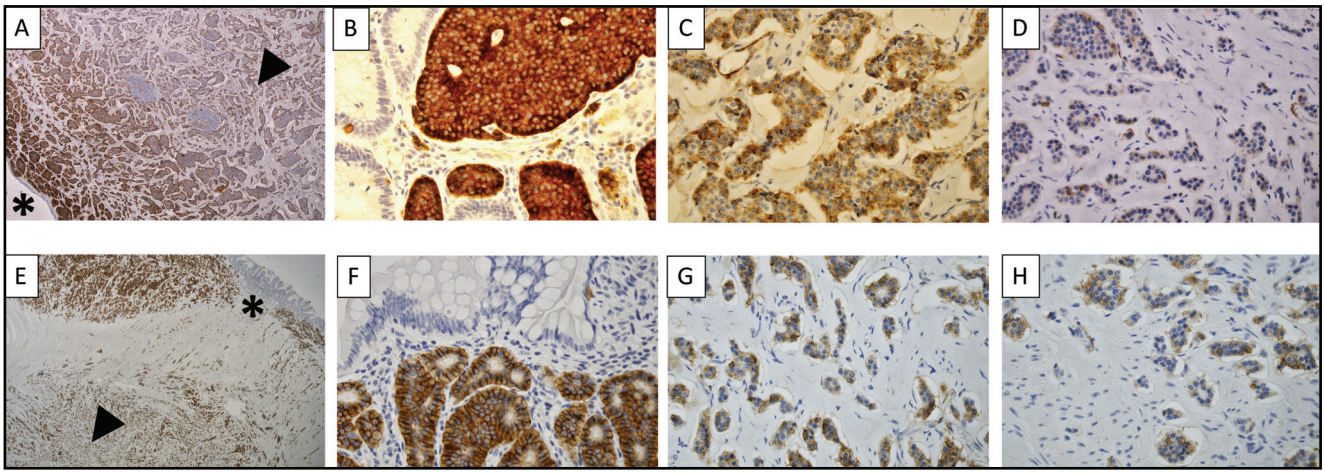

Fig. 1. Photomicrographs of the SSTR2 immunohistochemical stainings in SI-NETs. All images are magnified x400 unless otherwise specified. **A.** Case 8 displaying high SSTR2 expression (3+ membranous, 3+ cytoplasmic) in the mucosal region (asterisk) and low expression (0 membranous, 1+ cytoplasmic) in the underlying muscularis propria (arrowhead). Magnification x20. **B.** Case 17 displaying high SSTR2 expression (3+ membranous, 3+ cytoplasmic) in the mucosal region. **C.** Same case displaying low SSTR2 expression (0 membranous, 2+ cytoplasmic) in the muscularis propria region. **D.** Same case displaying low SSTR2 expression (0 membranous, 1+ cytoplasmic) in the subserosal region. **E.** Case 37 displaying high SSTR2 expression (3+ membranous, 3+ cytoplasmic) in the mucosal region (asterisk) and low expression (1+ membranous, 2+ cytoplasmic) in the underlying muscularis propria (arrowhead). Magnification x20. **F.** Same case displaying high SSTR2 expression (3+ membranous, 3+ cytoplasmic) in the mucosal region. **G.** Same case displaying low SSTR2 expression (1+ membranous, 2+ cytoplasmic) in the muscularis propria region. **H.** Same case displaying low SSTR2 expression (0+ membranous, 1+ cytoplasmic) in the subserosal region.

SSTR2 was significantly higher in the mucosal region compared to the muscularis propria and subserosal regions, where invading cells are acquiring genetic and epigenetic potential for early colonization, followed by intravasation and subsequent metastatic depositions (van Zijl *et al.* 2011).

This SI-NET primary cohort enabled us to investigate the oncologic role of SSTR2 in the very early stages of the disease development. Nevertheless, the rarity of the disease did not allow expanding the cohort in search for statistically significant analyses on patient survival. However, although based on a fairly small number of cases, our survival analysis demonstrated a trend for longer survival for patients with higher SSTR2 expression (Supplemental Figure 1). In addition, when scrutinizing cytoplasmic staining in the mucosal layer only, cases with low immunoreactivity displayed an increased risk of persistent disease at follow-up, indicating that this staining pattern could constitute a prognostic tool when assessing future risks for SI-NET patients.

In conclusion, this study suggests a physiological role for SSTR2 expression in SI-NETs, as the immunoreactivity across the histological layers of the small intestine is reduced concomitant with deeper tumor infiltration. This could signify that reduced levels of SSTR2 may be important for the invasive behavior of SI-NETs, and that SSTR2 immunoreactivity in clinical settings must be scrutinized in relation to the level of invasion in the small intestine. Our findings expand on the previous observations that SSTR2 immunoreactivity differs between primary tumors and metastases, and as shown here, the intensity might differ even across the histological regions within the same primary lesion. This could therefore affect the clinical interpretation regarding SSTR2 status in the tumor when investigating these tumors immunohistochemically as a part of the histopathological work-up, which in turn may have consequences in tailoring treatment for the patients.

ACKNOWLEDGEMENTS

This study was supported by grants provided from the Swedish Cancer Society, the Swedish Society for Medical Research and Radiumhemmets Forskningsfonder.

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