Original Paper

Evaluation of the expression of somatostatin receptors by immunohistochemistry in neuroendocrine tumors of the small intestine

Paweł Gut[1], Agata Czarnywojtek[2], Nadia Sawicka-Gutaj[1], Kosma Woliński[1], Małgorzata Janicka-Jedyńska[3], Katarzyna Bednarek-Rajewska[3], Katarzyna Furman[4], Marta Fichna[1], Marek Ruchała[1]

[1]Department of Endocrinology, Metabolism and Internal Medicine, Poznan University of Medical Sciences, Poznan, Poland
[2]Department of Pharmacology, Poznan University of Medical Sciences, Poznan, Poland
[3]Department of Clinical Pathology, Poznan University of Medical Sciences, Poznan, Poland
[4]Provincial Integrated Hospital L. Perzyny, Kalisz, Poland

Most neuroendocrine neoplasms (NEN) are characterized by the presence of somatostatin receptors (SSTR) which we use in location diagnostics and treatment. The aim of this study was to evaluate the expression of somatostatin receptors by immunohistochemistry in tissue obtained after surgery of the primary focus in the small intestine.

The group of patients consisted of 41 people, in 18 cases the primary tumor was in the jejunum and in 23 in the ileum. The immunohistochemical method was used to visualize the receptors, using polyclonal antibodies in a two-stage peroxidase method. In patients with NEN of the small intestine, the SSTR2a and SSTR5 receptors are most commonly expressed, followed by SSTR2b and 3. In statistical analysis, it was shown that the expression of somatostatin receptors was not dependent on the primary site of the tumor ($p > 0.05$). The dependence of SSTR expression on histological maturity is evident. SSTR1, SSTR2b, SSTR3 and SSTR5 are more common in tumors with grading G1 ($p < 0.05$). In the study group, the exception was SSTR2a, whose incidence was comparable in both groups ($p = 0.35$).

In NEN of the small intestine, the expression SSTR2a and SSTR5 is the most common.

Key words: somatostatin receptors, neuroendocrine tumor, small intestine.

Introduction

Neuroendocrine neoplasms (NENs) are unusual and relatively rare tumors that present many clinical challenges. Neuroendocrine neoplasms derive from diffuse neuroendocrine system cells, distributed in the gastrointestinal tract, pancreas and lungs. They characteristically synthesize, store and secrete a variety of peptides and neuroamines which can lead to the development of distinct clinical syndromes, including the carcinoid syndrome, however many are clinically silent until late presentation with mass effects. Classification of NENs was discussed regarding of their embryonal origin and secretory pattern with emphasis to the contemporary diagnostic procedures. A new classification has been developed by the WHO in 2017 dividing neuroendocrine neoplasms into well differentiated tumors G1 (Ki-67: below 3%), G2 (Ki-67: 3-20%), G3 (Ki-67: 21-55%) and poorly

differentiated carcinoma with proliferative index Ki- 67 more than 55% [1, 2]. Neuroendocrine neoplasms of the small intestine arise from the midgut region and most of them are hormonally active and produce serotonin. Neuroendocrine tumors are characterized by the presence of somatostatin receptors (SSTRs) on their surface [3, 4, 5, 6, 7, 8, 9]. Somatostatin receptors belong to the group of membrane receptors which have seven transmembrane loops and contain both extracellular and intracellular domains. These receptors are also associated with G protein (G protein-coupled receptor), and five subtypes of SSTRs (SSTR1–5) are identified. They are encoded by separate genes located on distinct chromosomes (SSTR1 on chromosome 14, SSTR2 on 17, SSTR3 on 22, SSTR4 on 20, and SSTR5 on 16) [10, 11, 12, 13]. The receptor subtype 2 occurs in two splicing variants, named as SSTR2a and SSTR2b. Recently, the occurrence of two additional SSTR5 variants has also been detected. In contrast to the classical form of the receptor, they contain five (SSTR5 TMD5) or four transmembrane domains (SSTR5 TMD4), respectively. The natural endogenous ligands for SSTRs are two molecular variants of somatostatin, composed of 14 and 28 amino acids. Both isoforms have high affinity for all SSTR subtypes. The treatment of neuroendocrine tumors with the somatostatin analogs is essentially based on inhibition of hormone secretion and cell proliferation, induction of apoptosis, and inhibition of angiogenesis [14, 15, 16, 17, 18, 19]. The largest study on the occurrence of various SSTR subtypes in neuroendocrine tumors, in which polymerase chain reaction (PCR) and immunohistochemistry were used in parallel, was presented by Papotti *et al.* [20]. In this study, 81 NEN cases were examined, including 28 gastrointestinal neoplasms and 53 pancreatic tumors. Their findings proved that the most commonly observed SSTR subtypes in NEN, detected in over 80% of the cases, were SSTR1 and SSTR2. SSTR3 and SSTR5 were present in 60% of the cases, while SSTR4 expression was rare. The expression of SSTRs was dependent on the histological grading of tumor tissue. Low-grade tumors with higher malignancy feature weak SSTR expression, and many authors have indicated the dominance of SSTR2 in NENs [21, 22]. Hubalewska-Dydejczyk *et al.* [23] investigated the presence of SSTR in gastrointestinal neuroendocrine tumors by immunohistochemistry, and SSTR2a was found to be present in all the examined tumors. SSTR5 was shown to be present in half of the examined tumors, whereas SSTR4 was not detected in any of the cases. Many studies have demonstrated variations in the location of particular SSTR subtypes; SSTR2a was mostly located in the membrane of the cell, while other subtypes were located in both cytoplasm and cellular membrane. The location of SSTR in the cellular membrane is an evidence for its preserved functionality. Reubi's study [24] indicates that the cytoplasmic location of the reaction is the result of internalization of the receptor under the influence of the agonist and depends on the concentration of somatostatin in the surrounding environment of tumor cells. The aim of this study was to evaluate the expression of SSTR subtypes (SSTR1, SSTR2a, SSTR2b, SSTR3, and SSTR5) in the tissue material obtained after surgery, where the primary lesions were located in the small intestine, by immunohistochemistry analysis.

Material and methods

The study group, in whom NENs were diagnosed in the small intestine, comprised 41 patients, including 29 women (70.7%) and 12 men (29.3%). At the beginning of the observation, the age of the subjects was in the range of 33-80 years (mean: 63.09 ±9.23 years). All subjects underwent surgical removal of the primary tumor with subsequent histopathological evaluation of the tumor tissue by the 2017 WHO classification system. In 18 patients, the primary focus was located in the jejunum, while in the remaining 23 cases it was located in the ileum (Table I). The study group was diagnosed and treated with somatostatin analogues in Endocrinology Department University of Medical Sciences, Poznan, Poland. Tumor tissue samples obtained from intestinal tumor after surgery were fixed in 10% buffered formalin (pH 7.4). The immunohistochemical technique was used to visualize the receptors, by incubating with appropriate polyclonal rabbit antibodies in a two-stage peroxidase method using the DAKO EnVision TM Flex kit (incubation with HRP – complex and Chromogen – complex). The results of the study were evaluated based on the immunoreactivity score (IRS) [25], which was calculated by multiplying the percentage of positive tumor cells with the staining intensity (Table II). Approval for the study (no. 969/10) was obtained from the Ethics Committee of the Poznań University of Medical Sciences.

Statistical analysis

The correlation between qualitative variables was analyzed by the χ^2 test or Fisher's test. A significance level of 0.05 was chosen for this analysis. Hence, all p-values > 0.05 were interpreted to indicate statistically significant dependencies. The analysis was carried out in the R program, version 3.3.1.

Results

In the group of patients with the primary location of the neuroendocrine tumor in the small intestine (grading G1 and G2), the SSTR2a and SSTR5

Table I. Characteristics of patients with small intestinal neuroendocrine neoplasms

Number	Patient	Gender (F/M)	Age (years)	Primary tumor	Liver involvement by metastasis (%)	Ki 67 (%)	Grading (G1/G2)
1.	K. K.	F	60	ileum	10	2	G1
2.	R .S.	M	65	ileum	10	2	G1
3.	G. L.	F	75	ileum	25	5	G2
4.	A. K.	F	53	jejunum	25	10	G2
5.	Z. C.	F	67	ileum	10	10	G2
6.	M. G.	M	60	jejunum	10	4	G2
7.	J. D.	F	73	jejunum	25	5	G2
8.	D. G.	F	70	ileum	10	10	G2
9.	J. B.	F	67	jejunum	10	5	G2
10.	M. A.	F	78	jejunum	25	10	G2
11.	Z. D.	F	55	ileum	10	1	G1
12.	Z. K.	M	60	ileum	10	4	G2
13.	A.G.	F	69	ileum	25	5	G2
14.	I. U.	F	49	ileum	25	10	G2
15.	J. W.	F	73	jejunum	25	10	G2
16.	A.W.	F	33	jejunum	10	4	G2
17.	B. S.	F	58	jejunum	10	2	G1
18.	J. S.	M	61	ileum	10	2	G1
19.	S. R.	M	65	ileum	10	2	G1
20.	S. S.	M	66	jejunum	25	5	G2
21.	J. S.	F	71	ileum	25	2	G1
22.	J. N.	F	68	ileum	10	2	G1
23.	K.P.	F	80	ileum	10	2	G1
24.	U. R.	F	67	ileum	10	2	G1
25.	A. S.	M	63	jejunum	10	2	G1
26.	B. R.	F	65	ileum	25	10	G2
27.	M. P.	F	71	ileum	10	1	G1
28.	Z. P.	M	66	jejunum	25	10	G2
29.	B. R.	F	67	jejunum	10	2	G1
30.	A. Ł.	M	51	ileum	10	2	G1
31.	R. P.	F	77	jejunum	25	10	G2
32.	E. W.	F	48	jejunum	10	2	G1
33.	K. D.	M	54	ileum	25	5	G2
34.	H. K.	F	65	jejunum	10	2	G1
35.	I. J.	F	67	ileum	25	5	G2
36.	Ł.T.	F	59	jejunum	25	5	G2
37.	J. W.	F	51	ileum	25	10	G2
38.	J. S.	M	57	jejunum	25	10	G2
39.	U. S.	F	61	ileum	10	2	G1
40.	M. T.	M	57	jejunum	25	10	G2
41.	E. K.	F	65	ileum	10	2	G1

Table II. Point scale of immunoreactivity – IRS

Percentage of positive cellular reactions	Intensity of color reaction	The IRS scale of immunoreactivity (0-12)
0 = lack of reaction	0 = lack of color reaction	0-1 = negative
1 = < 10% of positive cellular reactions	1 = weak reakction	2-3 = weak
2 = 10-50% of positive cellular reactions	2 = mild reaction	4-8 = mild
3 = 51-80% of positive cellular reactions	3 = strong	9-2 = strong
4 = > 80% of positive cellular reactions		
IRS – number of points		**IRS – classification**
0-1		0 = negative
2-3		1 = positive weak reaction
4-8		2 = positive mild reaction
9-12		3 = positive strong reaction

Point assessment of positive cell reactions and the intensity of color reaction in immunohistochemical evaluation of somatostatin receptors expression

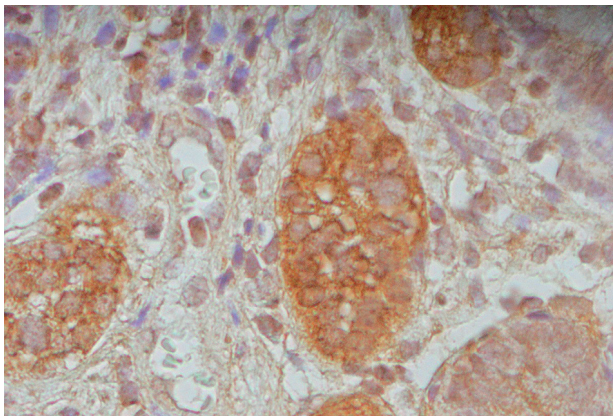

Fig. 1. Strong expression of SSTR2a (IRS score = 3) in ileum (cytoplasmic immunohistochemical reaction, 400×)

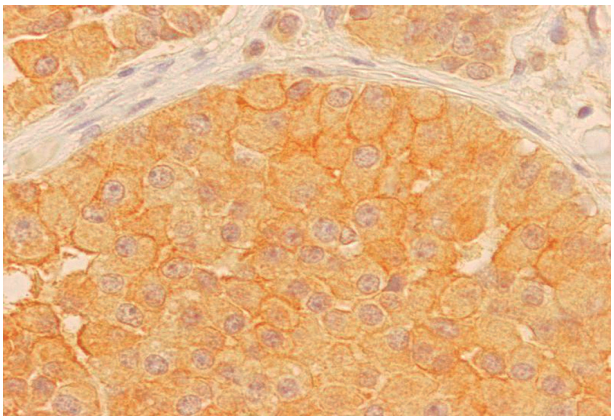

Fig. 2. Moderate expression of SSTR5 (IRS score = 2) in jejunum (membranous – cytoplasmic immunohistochemical reaction, 400×)

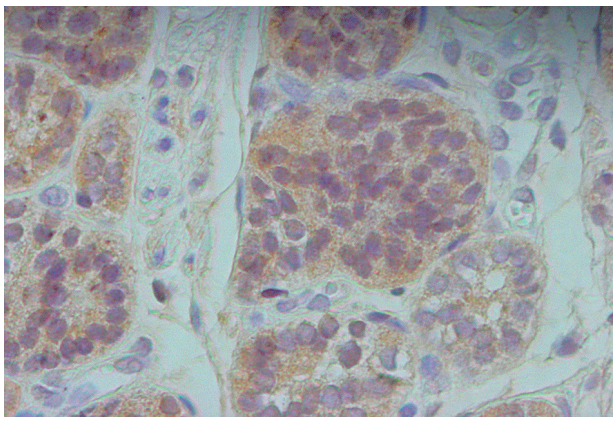

Fig. 3. Weak expression of SSTR1 (IRS score = 1) in ileum (cytoplasmic immunohistochemical reaction, 400×)

receptors were most commonly expressed, followed by SSTR2b and SSTR3, while the presence of SSTR1 was found to be least frequent. When the degree of expression of particular SSTR subtypes was assessed, applying the IRS classification system, strong SSTR expression (IRS score = 3) was most evident in the subtype SSTR2a (36.59%) (Fig. 1). Moderate SSTR expression (IRS score = 2) was most frequently observed in the subtypesSSTR2a (24.39%) and SSTR5 (21.95%) (Fig. 2).Weak SSTR expression (IRS score = 1) was noted in the subtypes SSTR2b (63.41%), SSTR1 (51.22%), and SSTR3 (46.34%) (Fig. 3). No expression of particular SSTR subtype (IRS score = 0) was most often observed in the subtypes of SSTR1 (48.78%) and SSTR3 (39.02%). In subsequent stages, the expression of particular SSTR subtypes and their intensities were evaluated, based on both the primary site (jejunum/ileum) and grading (G1 and G2) of the tumor.

Evaluation of the expression of somatostatin receptor subtypes according to primary tumor localization

The results regarding the frequency of expression of particular SSTR subtypes, in the group

Table III. The expression of SSTR receptors and primary tumor localization

Receptor	SSTR expression (primary localization)				p **
	Ileum (n = 18)		Jejunum (n = 23)		
	n	% *	n	% *	
SSTR1	8	44.44%	13	56.52%	0.651
SSTR2a	17	94.44%	19	82.61%	0.363 F
SSTR2b	9	50.00%	19	82.61%	0.059
SSTR3	10	55.56%	15	65.22%	0.759
SSTR5	12	66.67%	18	78.26%	0.489 F

** Percentages do not add up to 100% due to multiple-choice question.*

*** χ^2 test*

F Fisher's exact test (low expected values in the table)

SSTR – somatostatin receptors type 1, 2a, 2b, 3 and 5

Table IV. Expression of SSTR receptors according to grading

Receptor	SSTR expression (grading)				p **
	G1		G2		
	n	% *	n	% *	
SSTR1	16	84.21%	5	22.73%	< 0.001
SSTR2a	18	94.74%	18	81.82%	0.35 F
SSTR2b	19	100.00%	9	40.91%	< 0.001
SSTR3	16	84.21%	9	40.91%	0.012
SSTR5	19	100.00%	11	50.00%	0.001

** Percentages do not add up to 100% due to multiple-choice question.*

*** χ^2 test*

F Fisher's exact test (low expected values in the table)

SSTR – somatostatin receptors type 1, 2a, 2b, 3 and 5. Grading - histological maturity determined by the proliferative index Ki 67

of patients with tumor located in the jejunum, in the descending order are as follows: SSTR2a (94.44%), SSTR5 (66.67%), SSTR3 (55.56%), SSTR2b (50.00%), and SSTR1 (44.44%). In the group of patients with tumor located in the ileum, the expression pattern was found to be as follows: SSTR2a (82.61%), SSTR2b (82.61%), SSTR5 (78.26%), SSTR3 (65.22%), and SSTR1 (56.52 %). It is worth noting that both groups showed high expression of SSTR2a and low expression of SSTR1. However, the incidence of SSTR3 expression was comparable in both the groups. When the expression of SSTR2b and SSTR5 was analyzed, a slight predominance of these subtypes was observed in the group of patients in whom the tumor was localized within the ileum. Statistical analysis showed that despite the presence of small differences in the SSTR expression pattern, the expression of particular SSTR subtypes was not dependent on the primary tumor location ($p > 0.05$) (Table III).

Evaluation of the expression of particular somatostatin receptor subtypes according to grading

Among the patients with G1 tumors, SSTR5 and SSTR2b each (100.00%) and SSTR2a (94.74%) were most frequently observed, whereas SSTR1 and SSTR3 each (84.21%) were found to be the least frequent. In the group of patients with G2 tumors, the expression rates were significantly different and presented as follows: SSTR2a (81.82%), SSTR5 (50.00%), SSTR2b and SSTR3 each (40.91%), and SSTR1 (22.73%). The analysis clearly shows the dependence of SSTR expression on tumor grading, that is, SSTR1, SSTR2b, SSTR3, and SSTR5 were more commonly present in tumors with grading G1 ($p < 0.05$). In the study group, an exception with regard to the expression of SSTR2a was evident, the incidence of which was comparable in both the groups ($p = 0.35$) (Table IV).

Discussion

Consistent with the reports of other publications, the results of our study also demonstrated simultaneous presence of various SSTR subtypes in the same specimen [26, 27]. The findings of this study were in agreement with those reported for SSTR2a, which was observed to be the most frequently occurring SSTR subtype [28]. However, other authors reported that SSTR1 and SSTR5 were the most frequent subtypes of the SSTR [29]. Among the tumors examined in this study, the incidence rate of SSTR subtypes in a descending order was as follows: SSTR2a (87.80%), SSTR5 (73.17%), SSTR2b (68.29%), SSTR3 (60.98%), and SSTR1 (51.22). In the study group (n = 41), in whom the primary location of the tumor was the small intestine, SSTR2a and SSTR5 were the most common receptors, whereas SSTR2b, SSTR3, and SSTR1 were less frequent. Similar results were presented by Zamora *et al.* [30] for SSTR2a and SSTR5, where both subtypes were the most common. Diakatou *et al*. [31] presented the analysis of SSTR expression results in NENs, and the frequency of the mentioned receptors was as follows: SSTR2a (61.8%), SSTR2b (48.6%), SSTR1 (39.4%), SSTR3 (38.2%), SSTR5 (37.8%), and SSTR4 (15.4%). Volante *et al.* [32] analyzed 107 cases of neuroendocrine tumors, mainly evaluating the expression of SSTR2a, SSTR3, and SSTR5 subtypes. The results of the study indicated that for well-differentiated tumors, the incidence was SSTR2a (79%), SSTR5 (71%), and SSTR3 (44%), and for less differentiated neuroendocrine carcinomas the incidence was 44%, 28%, and 17%, respectively. In addition, it was observed that in the group treated with octreotide LAR there was 75% agreement between the results of SSTR2a expression and response to treatment [33]. This observation confirms the fact that SSTR2a is the main receptor subtype that has the potential to be used for imaging, diagnostics, and therapy.

In our study, we also performed a correlation analysis between histopathological grading and SSTR expression. In the G1 group, the most frequently expressed receptors were SSTR5 and SSTR2b (100%) and SSTR2a (94.74%), whereas in the G2 group, the incidence of the above receptor subtypes was lower and the following trend was observed: SSTR2a (81.82%), SSTR5 (50.00%), and SSTR2b (40.91%). Also, the incidence of SSTR1 and SSTR3 was lower in the G2 group. It is worth emphasizing that in a study that evaluated the relationship between the degree of SSTR expression and grading, the results showed a stronger association in the G1 group, which exhibited high IRS scores (2-3), while the scores were low (0-1) in the G2 group. Kim *et al*. [39] showed that well-differentiated NENs were characterized by more intense SSTR expression compared to tumors with a high mitotic index. Yerci *et al.* [34] reported a lack of correlation between the expression and grading of tumors with regard to SSTR2, while this relationship was demonstrated in the case of SSTR5, when using the same semi-quantitative assessment for evaluating the SSTR expression. There are also reports that NENs expressing SSTR2 and SSTR5 are characterized by a better prognosis, which can be explained by the fact that lesions with a lower Ki-67 proliferation index are characterized by better histological differentiation and higher expression of these receptors. The use of universal method for the assessment of SSTR expression, which includes the IRS classification system, however, has its limitations related to the subjectivity of evaluation, which may influence the final evaluation and comparison of test results. Therefore, the challenge was to compare the manual and automated analysis methods of SSTR expression [35]. Bad Berka Score1 is a virtual immunohistochemical staining score that compares the results of automatic evaluation method with those of the manual method, carried out using the technique of light microscopy. The analysis used includes SSTR1, SSTR2a, SSTR4 and SSTR5. IRS and Her2 scales were used in this study, which demonstrated a positive correlation between the automatic and manual methods with regard to SSTR2a and SSTR5 expression. Attempts were also made to compare the results obtained in this study with PET/CT imaging methods (^{68}Ga-DOTA-NOC), yielding quite promising results. The method proposed by German scientists seems to be effective and may, after a series of subsequent studies and modifications of the methodology, allow creating a standard for the evaluation of receptor expression by applying computer programs. It should be emphasized that further research is needed to assess the correlation between the expression of SSTRs, in particular SSTR2 and SSTR5, and the course of the disease, which would aid in both assessing the nature of the tumor and potential response to the therapy. The gold standard in the treatment of carcinoid syndrome symptoms are currently somatostatin analogues, which have high affinity for somatostatin receptors (mostly SSTR2a and SSTR5) located on the tumor surface. The most commonly used preparations with a long duration of action are octreotide and lanreotide, which have the highest affinity for the SSTR2 and SSTR5 receptor, and less so for SSTR3, and no affinity for SSTR1 and SSTR4. A very important element in qualifying for treatment with SSA analogs is the evaluation of the expression of these receptors using whole body receptor scintigraphy (^{99}mTc-EDDA/HYNIC-TOC and PET/CT using ^{68}Ga-DOTA/TATE), or an immunohistochemical assessment of the occurrence of SSTR in tissue material collected during surgery or biopsy [36,

37, 38, 39, 40]. Summing up, it should be stated that the assessment of somatostatin receptor expression in neuroendocrine tumors is a key element in the planning of diagnosis and treatment.

The authors declare no conflict of interest.

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Address for correspondence

Paweł Gut
Department of Endocrinology, Metabolism and Internal Medicine
Poznan University of Medical Sciences
Przybyszewskiego 49
60-355 Poznan, Poland
e-mail: gutpj@poczta.onet.pl