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Determination of Somatostatin Receptor Subtype 2 in Carcinoid Tumors by Immunohistochemical Investigation with Somatostatin Receptor Subtype 2 Antibodies[1]

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ABSTRACT

We have shown previously that expression of mRNA for somatostatin receptor subtype 2 (sst_2) detected by *in situ* hybridization correlates to therapeutic outcome in patients with carcinoid tumors treated with somatostatin analogues. However, *in situ* hybridization is laborious and not practical in clinical routine work. We have, therefore, developed polyclonal antibodies directed against sst_2 that may be used for immunohistochemistry on tissue specimens. The staining is specific and is highly correlated to expression of mRNA for sst_2 ($P < 0.01$) as well as to tracer uptake at somatostatin receptor scintigraphy ($P < 0.01$). There is also a good correlation to the therapeutic response in carcinoid patients treated with somatostatin analogues ($P < 0.05$). Of 35 patients with carcinoid tumors included in this investigation, 25 stained positive with the antibodies. Twenty-two of these were investigated by somatostatin receptor scintigraphy and showed tracer uptake in metastases. An additional two patients that did not stain with the antibodies showed pathological uptake of the tracer in metastases, which might indicate binding to somatostatin receptor subtype 5. None of the 10 patients without positive immunostaining responded to somatostatin analogue treatment, whereas patients with a positive stain had a biochemical response or remained stable during treatment. Thus, these antibodies may be used to determine the presence of sst_2 in carcinoid tumors and to select patients suitable for somatostatin analogue treatment. The method is easily applicable in clinical practice.

INTRODUCTION

ssts[3] and their ligand somatostatin are widely distributed throughout the body and are thought to participate in such different regulatory systems as inhibition of hormone secretion from endocrine cells, growth regulation, and induction of apoptosis (1, 2). Five different subtypes of ssts have been cloned (3–5). The somatostatin analogues available for clinical use bind with high affinity to sst_2 and sst_5 and with medium affinity to sst_3 (6). The expression of the five sst subtypes seems to be different in different tissues, and this variability may account for the diverse actions exerted by the receptor-ligand complex (2).

It is well known that ssts are expressed in neuroendocrine tumors (7). This is the molecular bases for somatostatin analogue treatment in patients with such tumors. During somatostatin analogue treatment, hormone levels are reduced, and symptoms related to hormone production decrease (8). Tumors expressing sst may also be visualized by sst scintigraphy. During scintigraphy, the radioactive compound [^{111}In-DTPA-D-Phe^1]octreotide is accumulated in tissue expressing ssts, which bind octreotide, and the result can be correlated to the patient's response to somatostatin analogue treatment. However, because octreotide can bind to different ssts, we cannot tell which subtype is expressed by the tumor, and some patients display tracer uptake but lack the biochemical response (9).

We have shown previously that expression of sst_2 seems to be a prerequisite in patients with carcinoid tumors treated with somatostatin analogues to be able to respond by a reduction in hormone levels (10). Similar results concerning tumor growth have been shown for pancreatic cancer cell lines (11).

Patients with malignant neuroendocrine tumors suffer from symptoms induced by hormones that are produced by the tumor cells. In patients with carcinoid tumors, a carcinoid syndrome develops consisting of flushes, diarrhea, carcinoid heart disease, and bronchial constriction. These symptoms may be severe enough to prevent the patient from performing normal daily activities, and treatment with somatostatin analogues may abolish or reduce the symptoms. However, in patients without expression of sst_2, hormone levels may remain unchanged or even increase during treatment with somatostatin analogues, and there is no clinical amelioration.

Until now, the only methods reported to detect which subtype of sst that is expressed has been by *in situ* hybridization or PCR methods. These methods are laborious and not practical for clinical routine applications. Because treatment with somatostatin analogues is expensive and of no value if expression of sst_2 is lacking, it is important to determine the sst_2 content in each tumor. Development of antibodies specific for the different ssts have thus far only provided compounds that may be used on Western blots and for immunoprecipitation (12). Our aim has, therefore, been to develop sst subtype-specific antibodies that may be used for immunohistochemistry on tissue sections. Such a method would provide a reliable overnight answer to the question of whether the patients' tumor cells express sst_2. In this study, we report on the development of such antibodies for sst_2 and the initial clinical results.

MATERIALS AND METHODS

Development of Antibodies. After making a deduction from the amino acid sequences of human sst_2, a polypeptide was synthesized by a solid-phase system using Fmoc chemistry (Applied Biosystems model 430 A, Foster City, CA). Peptides were purified by reverse-phase chromatography and analyzed by plasma desorption mass spectrometry (PDMS, Bioion 20; Bioion Nordic AB, Uppsala, Sweden). The sequence was selected to be specific for sst_2, and the homology was <44% to any other known protein sequence in the databank MPsrch Protein, version 1.5 (Shane S. Surrock and John F. Collins, Biocomputing Research Unit, University of Edinburgh, United Kingdom), except from the sequence of sst_2 from other species. The selected amino acid sequence for sst_2 was amino acids 330–343 with an additional tyrosine residue at the NH_2-terminal end (Tyr_0) and an amidated COOH-terminal (3). Before immunization, the peptide was coupled to a carrier protein. Two mg of the peptide and 20 mg of bovine serum albumin were dissolved in a 50 mM sodium phosphate buffer of pH 7.4 containing 150 mM sodium chloride. Coupling was then induced by addition of 90 μl of glutaraldehyde (13). The resulting complexes were injected into rabbits, using the multiple intradermal injection technique to produce polyclonal antibodies (14).

Affinity Purification of the Antibodies. The synthesized peptide, 3 mg, was coupled to 2 g of an activated agarose gel (Novarose Act-High 10000/40;

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[3] The abbreviations used are: sst, somatostatin receptor, sst_2, sst subtype 2.

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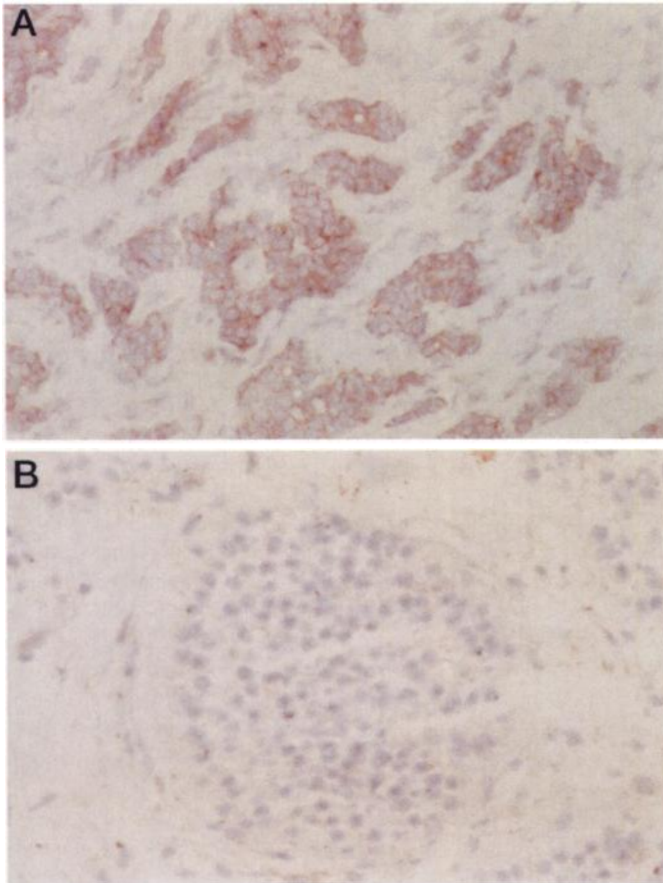

Fig. 1. *A*, immunohistochemical staining of a midgut carcinoid tumor with the polyclonal antibody against sst_2. The tumor cells are strongly stained at the cell surface. This patient had a positive sst scintigraphy and responded to somatostatin analogue treatment. *B*, immunohistochemical staining of a midgut carcinoid with the polyclonal antibody against sst_2. In this patient, the tumor cells failed to stain positive. This patient also had a negative sst scintigraphy.

Inovata, Bromma, Sweden) according to the manufacturer's instructions and packed in a disposable column. One ml of rabbit antiserum was diluted with one ml of PBS and applied upon the column. The column was than washed with 5 ml of PBS, and the affinity-coupled antibodies were eluted with 5 ml of 0.1 M glycine-buffer at pH 3.0 containing 0.15 M sodium chloride.

Cell Lines. Control cell lines stably expressing different ssts were made using CHO-K1 cells. cDNAs for the five different ssts were cloned in expression plasmid pcDNA3 (InVitrogen, San Diego, CA). Plasmids were prepared, and 1–2 μg were used for transfection of 105 CHO-K1 cells using the Lipofectin reagent (Life Technologies, Inc., Gaithersburg, FL). After 48 h of culture, G418 was added to a final of concentration of 700 $\mu g/ml$. Selection of transfectants proceeded for a total of 14 days. As a control, CHO-K1 cells transfected with pcDNA3 vector alone were used.

Tissue Material. Tumor tissue from 35 patients with histopathologically verified carcinoid tumors were included for immunohistochemical examination. Tumor samples obtained at operation or by ultrasound-guided needle biopsies (1.2 mm) from metastases were frozen in liquid nitrogen and kept at −70°C until sectioned. Thirty-two patients had been examined by sst scintigraphy (OctreoScan).

Immunohistochemistry. After cryosectioning, tissue was fixed in 100% acetone for 10 min and then washed twice in PBS. Endogenous peroxidase was blocked with 0.3% hydrogen peroxidase in PBS, and avidin-binding protein was blocked by incubating the sections sequentially with avidin and biotin in Blocking kit (Vector Laboratories, Burlingame, CA). The affinity-purified rabbit polyclonal antibodies were used as primary antibody and diluted 1:20 in PBS with 1% bovine serum albumin, pH 7.4. The antibodies were applied over night at 4°C. The immunoreaction was visualized with an Elite kit (Vector Laboratories), and 0.02% hydrogen peroxidase was used as substrate and 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO) in dimethylsulfoxide as chromogen. The sections were also counterstained with hematoxylin.

***In Situ* Hybridization.** The plasmid containing cDNAs encoding for sst_2 was a kind gift from Dr. G. Bell (University of Chicago, Chicago, IL) and has been described previously (3). Antisense and sense cRNA probes were transcribed from linearized plasmids using [α-^{35}S]UTP (>1000 Ci/mmol; Amersham, Buckinghamshire, United Kingdom) and T3 or T7 RNA polymerase (Boehringer Mannheim, Mannheim, Germany) as described before (15). The integrity of mRNA in the tissue sections was checked with a [α-^{35}S]UTP-labeled β-actin cRNA probe transcribed from a 1.7-kb cDNA with SP6 RNA polymerase (Boehringer; Ref. 16).

In situ hybridization was carried out as described before (15) and could be performed in 26 patients. Briefly, the slides were treated with acetic anhydride followed by Tris-HCl buffer containing glycine. About 0.5–1 ng of probe in 20-μl probe solution was hybridized to cells overnight at 50°C in an humidified chamber. The slides were then washed and treated with RNAse A, washed again, dehydrated in ethanol, and finally autoradiographed with NTB-2 nuclear track emulsion (Eastman Kodak, Rochester, NY) for 14 days. The developed slides were counterstained with hematoxylin solution and examined in a light microscope at a magnification of ×400. The examination and assessment was performed blindly by one investigator without knowledge of the patient's identity.

Medical Treatment. Outcome of medical treatment with a somatostatin analogue was evaluable in 21 of the 35 patients. Patients were treated with different analogues as indicated in Tables 1–3. The remaining 14 patients were treated either with interferon or a combination of interferon and a somatostatin analogue, and these patients could therefore not be evaluated for biochemical response with respect to somatostatin analogue treatment.

Response Criteria. A partial biochemical response (PR) was defined as a decrease in biochemical markers (Chromogranin A or U-5HIAA) by >50%. Biochemical progress was defined as an increase in biochemical markers by >25%. Stable disease was defined as a decrease in biochemical markers by <50% or an increase by <25%.

Statistics. Fisher's exact test was used.

RESULTS

Purified antibodies were initially tested on different CHO-K1 cell lines including wild-type not expressing any sst, cells transfected with one of the five sst subtypes, and cells transfected with the vector only. The antibodies only stained the CHO-K1 cells transfected with sst_2, whereas all other cell lines remained unstained (data not shown).

When the antibodies were applied on tumor specimens from patients with carcinoid tumors, we found that they stained mainly the surface of the tumor cells (Fig. 1*A*). Tumor tissue from five patients with bronchial carcinoid tumors was stained, and only one of these patients stained positive with the sst_2 antibodies, whereas the other four were negative. This result correlated well to the outcome of sst scintigraphy; the only patient with a positive stain at immunohistochemistry also showed uptake at scintigraphy. This patient had a biochemical response when treated with octreotide, whereas the other two patients treated with somatostatin analogues showed negative immunostaining, lacked expression of mRNA for sst_2, and were nonresponders with increasing hormone levels during treatment (Table 1).

Table 1 *Clinical data on patients with foregut carcinoid tumors*

Patient no.	Immuno-histochemistry[a]	*In situ* hybridization	Octreoscan	Somatostatin analogue treatment	Response[b]
1	Pos	ND	Pos	Octreotide	PR
2	Neg	Neg	Neg	Octreotide	PD
3	Neg	Neg	Neg	Somatuline	PD
4	Neg	Neg	Neg	ND	
5	Neg	ND	Neg	ND	

[a] Pos, positive; Neg, negative.
[b] PR, partial response; PD, progressive disease; ND, not done.

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Among the 30 patients with midgut carcinoid tumors, tissue specimen from 24 patients stained positive for sst_2 (Fig. 1*A*), whereas the remaining 6 were negative (Fig. 1*B*; Tables 2 and 3). The correlation to tracer uptake at OctreoScan was also good, although 2 of the 6 patients that did not stain with the sst antibody actually had a positive OctreoScan. One of these patients had expression of mRNA for sst_2, whereas one patient who stained with the antibodies also lacked expression of sst_2 mRNA. All patients with positive staining for sst_2 had tracer uptake in tumor lesions at OctreoScan.

Overall in the 32 patients investigated by both immunohistochemistry and sst scintigraphy, the correlation between the outcome of the two investigations was very high ($P < 0.01$).

In situ hybridization for expression of sst_2 mRNA in the 26 patients that could be evaluated showed a high correlation to immunostaining with the antibody against sst_2 ($P < 0.01$).

Of the midgut carcinoid patients, 18 could be evaluated for biochemical response to somatostatin analogue treatment, and in this group, 9 responded with at least 50% reduction in hormone levels during treatment. All of these patients stained positive with the antibody and had tracer uptake at OctreoScan. Six patients had stable biochemical levels during treatment, and all of these also stained positive with the antibodies and had tracer uptake at OctreoScan. In three patients, biochemical levels increased, and none of these stained positive with the antibody. However, one of these patients showed tracer accumulation at OctreoScan, whereas the other two did not (Table 3).

The correlation between biochemical response and immunostaining in the 21 patients that were treated with somatostatin analogues was significant ($P < 0.05$).

DISCUSSION

We have developed polyclonal antibodies recognizing sst_2, which may be used for immunohistochemical staining of tissue. These antibodies are specific for sst_2, and there is no cross-reaction with the other ssts.

Patients with neuroendocrine tumors are responsive to treatment with somatostatin analogues, and there is usually a decrease in symptoms and hormone levels during treatment. It has also been argued that somatostatin analogues may be involved in growth inhibition, and it has been proposed that sst_3 may be involved in the induction of apoptosis, whereas sst_1 and sst_2 may be involved in other pathways for growth inhibition (2). However, in clinical materials, objective tumor responses with significant reduction in tumor size are very rare, seen in only about 5% (8, 17). Biochemical response is, therefore, the main therapeutic response during somatostatin analogue treatment and mediated predominantly through sst_2. sst scintigraphy may also be used to select patients suitable for somatostatin analogue treatment; however, in up to 20% of the patients, OctreoScan may be positive, but the patients lack expression of sst_2 mRNA or fail to respond to somatostatin analogue treatment (9, 10). This might be explained by the fact that the analogue used for the scintigraphic investigation also binds to sst_3 and sst_5. Therefore, determination of sst_2 is of very high clinical importance. If there is an expression of sst_2, one should expect a good response to somatostatin analogue treatment in terms of hormone reduction and improvement of symptoms, as we and others have shown by *in situ* hybridization or PCR methods (10, 18). These methods are laborious and therefore not easily applied in routine clinical work. Because somatostatin analogue treatment is expensive and has to be administered two or three times daily, it is of clinical importance to determine the presence of sst_2 before treatment is started. Patients without sst_2 may then be subjected to alternative active treatment, such as interferon-α.

We are also expecting new somatostatin analogues in the future that are specific for other ssts, and this is another indicator of the importance to find out which subset of ssts is present in an individual patient (19).

The antibody that we have developed may be used for immunohistochemical staining of tumor tissue. Immunohistochemical staining was strongly correlated with expression of mRNA for sst_2 in the tumor cells ($P < 0.01$). When the result from the staining was correlated with other clinical data, we found that the correlation to tracer uptake at OctreoScan was high ($P < 0.01$), and there was also a significant correlation to therapeutic response ($P < 0.05$). Thus, these antibodies are at least as good as the sst scintigraphy in detecting tumors with sst_2 expression.

We are now in the process of developing antibodies against the other four ssts. Our hope is that in the near future, we may use sst scintigraphy for staging of patients with neuroendocrine tumors, while we may use the antibodies to reveal which subset of receptors that is present. The latter is of importance for the individualization of the medical treatment for these patients.

Table 2 *Clinical data on patients with midgut carcinoid tumors and positive immunohistochemical staining for sst_2*

Patient no.	Immuno-histochemistry[a]	*In situ* hybridization	Octreoscan	Somatostatin analogue treatment	Response[b]
1	Pos	Pos	Pos	Octreotide	PR
2	Pos	Pos	Pos	Octreotide	PR
3	Pos	Pos	Pos	Octreotide	PR
4	Pos	ND	Pos	Octreotide	PR
5	Pos	Pos	Pos	Octreotide	PR
6	Pos	Pos	Pos	Octreotide	PR
7	Pos	Neg	Pos	Octreotide	SD
8	Pos	Pos	Pos	Octreotide	SD
9	Pos	Pos	Pos	Octreotide	SD
10	Pos	ND	Pos	Octreotide	SD
11	Pos	ND	Pos	Octreotide	SD
12	Pos	Pos	Pos	Octastatin	PR
13	Pos	Pos	Pos	Octastatin	PR
14	Pos	Pos	Pos	Somatuline	PR
15	Pos	ND	Pos	Somatuline	SD
16	Pos	Pos	Pos	ND	
17	Pos	Pos	Pos	ND	
18	Pos	Pos	Pos	ND	
19	Pos	Pos	Pos	ND	
20	Pos	ND	Pos	ND	
21	Pos	Pos	Pos	ND	
22	Pos	ND	ND	ND	
23	Pos	Pos	ND	ND	
24	Pos	Pos	ND	ND	

[a] Pos, positive; Neg, negative.
[b] PR, partial response; SD, stable disease; ND, not done.

Table 3 *Clinical data on patients with midgut carcinoid tumors without positive immunohistochemical staining for sst_2*

Patient no.	Immuno-histochemistry[a]	*In situ* hybridization	Octreoscan	Somatostatin analogue treatment	Response[b]
1	Neg	Neg	Pos	Octreotide	PD
2	Neg	Neg	Pos	ND	
3	Neg	Neg	Neg	Octreotide	PD
4	Neg	ND	Neg	Octreotide	PD
5	Neg	Pos	Neg	ND	
6	Neg	ND	Neg	ND	

[a] Neg, negative; Pos, positive.
[b] PD, progressive disease; ND, not done.

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