A Comparison between the Efficacy of Somatostatin Receptor Scintigraphy and That of in Situ Hybridization for Somatostatin Receptor Subtype 2 Messenger RNA to Predict Therapeutic Outcome in Carcinoid Patients

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ABSTRACT

Predictive tests for treatment with somatostatin analogues have been asked for by clinicians. We have shown previously that somatostatin receptor (sstr) scintigraphy may be used to predict therapeutic outcomes for carcinoid patients receiving somatostatin analogues. However, almost 28% of patients with pathological tracer uptake fail to respond to such treatment. To increase further the reliability and prognostic value of sstr identification, we investigated the presence of mRNA for the subtypes sstr1 and sstr2 by in situ hybridization on tumor specimen from 25 carcinoid patients (22 midgut, 2 foregut, and 1 hindgut), all receiving somatostatin analogue treatment (12 lanreotide, 8 octreotide, and 5 octotatin) and compared this to the therapeutic response evaluated as inhibition of hormone secretion. Expression of sstr2 mRNA could be detected in 15 patients, all responding to somatostatin analogue treatment and showing pathological tracer uptake in tumor lesions at sstr scintigraphy. In the remaining 10 patients, no sstr mRNA could be detected, and none of the patients responded to somatostatin analogue treatment. Three of these 10 patients failed to accumulate tracer activity at sstr scintigraphy, whereas 7 had a pathological uptake of \( [^{111}\text{In-DTPA-D-Phe}_{1}]\)-octreotide. We conclude that in this group of carcinoid patients, there was complete agreement between the presence of mRNA for sstr2 detected by in situ hybridization and therapeutic outcome. In patients with pathological tracer accumulation without expression of somatostatin sstr2 mRNA, other sstr may be present that can bind the somatostatin analogue but not inhibit hormone secretion.

INTRODUCTION

Carcinoid tumors are slowly growing neoplasms that may produce large amounts of hormones such as serotonin and tachykinins, including substance P and neuropeptide K (1). Patients with active hormone production suffer from symptoms such as diarrhea and flushes, which are part of the carcinoid syndrome (2). These symptoms may be severe enough to stop the patient from normal social activities.

The primary treatment for these patients is surgery, but because the tumors may have an indolent course, many patients have liver metastases already at diagnosis (3). Thus, medical treatment aiming at tumor reduction and symptomatic relief is of utmost importance. Chemotherapy has not been very successful with responses of short duration in 10–30% of patients treated (4, 5). The introduction of biotherapies, \( \alpha \)-IFN (6), and somatostatin analogues (7) have contributed to an improvement in the treatment of these patients, including both reduction in tumor growth and relief of symptoms.

Somatostatin is distributed widely throughout the body, and its main function is to inhibit the release of hormones from endocrine cells (8). This mode of action is mediated through specific receptors present on the cell surface. It has been shown that carcinoid tumors express high amounts of sstr3 on the cell surface (9). During the last few years, five different subtypes of sstrs have been cloned (10–12). It is known that the somatostatin analogues used most frequently in the clinic binds preferentially to sstr2, sstr3, and sstr5 (13). Until now, somatostatin analogues have been used to decrease symptoms induced by hormones produced by the carcinoid tumors. This action of somatostatin analogues is mediated through sstr2 (14). It has also been shown that the growth-inhibiting action of somatostatin analogues is mediated through different mechanisms by sstr1, sstr3, and sstr5 (15, 16). However, the clinical significance of this growth inhibition still has to be evaluated.

A new technique for tumor visualization utilizing the radioactive compound \( [^{111}\text{In-DTPA-D-Phe}_{1}]\)-octreotide has been developed (17) and has become frequently used in the diagnosis and staging of patients with neuroendocrine tumors. We have shown previously that the presence of sstrs in carcinoid tumors detected by sstr scintigraphy may predict the therapeutic response to somatostatin analogues in carcinoid patients (18). However, in our study, 18% of the patients with sstr-positive tumors detected by sstr scintigraphy failed to respond by means of hormone reduction to somatostatin analogue treatment. The reason for this is unclear, but may at least in part be due to binding of the \( [^{111}\text{In-DTPA-D-Phe}_{1}]\)-octreotide complex to subtypes of sstrs that do not mediate inhibition of hormone secretion.

Kubota et al. (19) have shown that presence of sstr2 mRNA could be correlated to response to somatostatin analogue treatment in one patient with a glucagonoma, whereas another patient with a carcinoid tumor lacking this mRNA expression was unable to respond to such treatment. Because of these interesting data in individual patients, we decided to perform a clinical study of the predictive value of mRNA detection for sstr1, an sstr subtype not known to inhibit hormone production, and sstr2, which is known to inhibit hormone release, in patients with carcinoid tumors referred for medical treatment with a somatostatin analogue.

MATERIALS AND METHODS

Patients. We have included 25 patients, 11 males and 14 females with histopathologically confirmed carcinoid tumors referred for medical treatment between 1992 and 1994 to the Department of Internal Medicine, Endocrine Oncology Section, in this study. There were 22 patients with midgut, 2 with foregut, and 1 with hindgut carcinoid tumors. The median age was 60 years (range, 47–79 years). All patients had malignant tumors with liver metastases and elevated hormone levels. Nineteen of the patients had a carcinoid syndrome with flushes and/or diarrhea. Eleven of these patients were included in our previous study concerning the correlation between the outcome of sstr scintigraphy and biochemical response to somatostatin analogue treatment (18). Tumor tissue for in situ hybridization and immunohistochemical staining was obtained either during surgery or by ultrasound-guided needle biopsies (1.2 mm) from liver or lymph node metastases before treatment was started. Tumor tissue was frozen in liquid nitrogen and stored at \(-70^\circ\text{C}\). All patients, except the one with a hindgut carcinoid tumor, had elevated levels of urinary 5-hydroxyindoleacetic acid. The hindgut carcinoid patient had an increased

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: sstr, somatostatin receptor; SPECT, single-photon emission computed tomography; DTPA, diethylenetriamine pentaacetic acid.
level of human chorionic gonadotrophin α subunit. Patients were monitored every third month using the tumor markers urinary 5-hydroxyindoleacetic acid, which was calculated as the mean of two 24-h collections (determined according to a method described earlier: Ref. 20) or, in the case of the patient with a hindgut carcinoid tumor, human chorionic gonadotrophin α subunit levels in serum (21). An objective response was defined as a decrease in tumor markers by ≥50%. Patients with less reduction in tumor markers were assessed as nonresponders.

**Treatment.** Patients included were subjected to treatment with a somatostatin analogue. The outcome of the in situ hybridization was not known before treatment was started. Eight patients were treated with octreotide (Sandoz Pharma Ltd., Basel, Switzerland), 12 with lanreotide (Ipsen Biotec ApS, Copenhagen, Denmark), and 5 with octastatin (Debiopharm SA, Lausanne, Switzerland). Octreotide was administered as a s.c. injection twice daily at a median dose of 200 μg/day. Lanreotide was either given as a s.c. injection four times daily with a daily dose of 6,000–12,000 μg (n = 11) or as an i.m. injection of lanreotide LAR (a long-acting formula) at a dose of 30 mg every second week. Octastatin was administered as a continuous s.c. infusion at a dose of 3,000 μg daily using a micropump (Debiotech SA, Switzerland).

**Somatostatin Receptor Scintigraphy.** The sstr scintigraphy was performed as described earlier (22). Patients were injected i.v. with 100–200 MBq of $^{111}$In-DTPA-octreotide (Mallinkrodt Medical of Petten, The Netherlands). At 24 h after injection, static anterior-posterior whole-body images were collected, and a SPECT was performed over the area of interest using a γ scintillation SPECT camera equipped with a medium-energy general purpose collimator (Nuclear Diagnostics, Hägersten, Sweden). The collection of original data for the SPECT images was performed using a 64-step rotation of 360° in a 64 × 64-word matrix. Energy windows of 173 and 247 keV (±10%) were used. The collection time for each angle was 40 s, giving a total of about 30,000 counts/angle. For the reconstruction of SPECT images, a Wiener filter was applied to the original data.

**In Situ Hybridization.** In situ hybridization was performed on tissue obtained as described above. Tissue samples were cryosectioned and fixed in 4% paraformaldehyde for 3 min at room temperature. Slides were stored in 70% ethanol at 4°C until use.

The plasmids containing CDAs encoding for sstr, and sstr were a kind gift from Dr. G. Bell (University of Chicago) and have been described previously (10). Antisense and sense cRNA probes were transcribed from linearized plasmids using α-35S-labeled UTP (>1000 Ci/mm; Amersham, Little Chalfont, UK) and T3 or T7 RNA polymerase (Boehringer Mannheim, Mannheim, Germany) as described before (23). The integrity of mRNA in the tissue sections was checked with a α-35S-UTP-labeled β-actin cRNA probe transcribed from a 1.7-kb cDNA with SP6 RNA polymerase (Boehringer Mannheim; Ref. 24).

In situ hybridization was carried out as described before (23). Briefly, the slides were treated with acetic anhydride followed by Tris-HCl buffer containing glycine. About 0.5–1 ng probe in 20 μl probe solution was hybridized to cells overnight at 50°C in a humidified chamber. The slides were then washed and treated with RNase A, washed again, dehydrated in ethanol, and 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 patient identity.

**Immunohistochemistry.** Consecutive cryosections from the same tissue samples as were used for in situ hybridization were also stained with H&E and with a polyclonal antibody against chromogranin A and B to show the presence of tumor cells (25). For the immunohistochemical procedure, the Vectastain avidin-biotin complex method kit (Vector Laboratories, Burlingame, CA) was used with 3-aminon-9-ethylcarbazole as chromogen.

**RESULTS**

The results are summarized in Table 1. Of the 25 patients included in this study, 15 (60%) had expression of mRNA for sstr2; of these, 12 also had expression of sstr2 mRNA. All 15 patients responded in terms of hormone reduction to somatostatin analogue treatment and had pathological tracer accumulation in tumor lesions at sstr scintigraphy (Fig. 1). Of these 15 patients, 13 had symptoms of a carcinoid syndrome with flushes and/or diarrhea, and, of these, 8 experienced relief of their symptoms.

The remaining 10 patients without sstr2 mRNA expression all failed to respond to somatostatin analogue treatment. Of these patients, five were treated with lanreotide at a dose of 12,000 μg daily, which is a high dose; one received lanreotide LAR; two were treated with octastatin at a dose of 1,500 μg daily (all eight patients received treatment according to pre-fixed dose escalation schedules); and two patients were treated with octreotide. In the octreotide-treated patients, dose escalation did not improve the therapeutic result. Of these 10 patients, 7 had flushes and/or diarrhea, and only one experienced a relief of the symptoms during treatment with a somatostatin analogue. Five patients lacked expression of both sstr1 and sstr2, whereas five expressed sstr1 mRNA. Seven patients had a pathological tracer accumulation in tumor lesions at sstr scintigraphy, whereas three patients had no tracer uptake. Of the three patients without $^{111}$In-DTPA-octreotide accumulation, two expressed sstr1 mRNA, and one had no expression of mRNAs analyzed in the study. In four patients, sstr1 and sstr2 mRNA could not be detected, although sstr scintigraphy was positive.

Neither of the two patients with foregut carcinoid tumors had sstr2 mRNA expression or $^{111}$In-DTPA-octreotide accumulation in tumor lesions, and both were treatment failures (Fig. 2). However, both expressed sstr1 mRNA. The patient with a hindgut carcinoid tumor showed expression of both receptor subtypes, had tracer accumulation at sstr scintigraphy, and responded to somatostatin analogue treatment.

**DISCUSSION**

The basis for somatostatin analogue treatment in carcinoid patients is the presence of sstrs on tumor cells. It has been shown that the presence of mRNA for sstr may be correlated to the responsiveness of a patient to somatostatin analogue treatment (19). However, this has only been shown in individual patients, and additional analyses are needed. We have shown previously that sstr scintigraphy may be used to exclude patients lacking $^{111}$In-DTPA-octreotide.
uptake from somatostatin analogue treatment (18). However, in our study, 18% of the patients with $[^{111}\text{In-DTPA-o-Phe}^1]$-octreotide accumulation in tumor lesions failed to respond to subsequent somatostatin analogue treatment. The reason for this was previously unclear, and defect receptors or impaired intracellular signaling were suggested.

In this study, we have found complete agreement between the presence of sstr$_2$ mRNA and the outcome of somatostatin analogue treatment in terms of inhibition of hormone secretion. All patients expressing this mRNA also responded to somatostatin analogue treatment. Such a correlation has also been suggested by others (19, 26). However, the presence of sstr$_2$ mRNA and outcome of sstr scintigraphy was inconsistent. In seven (28%) patients, there was a pathological tracer uptake, although no sstr$_2$ mRNA could be detected. It seems probable that tracer accumulation in tumor lesions is not only dependent on the presence of sstr$_2$, but other sstrs may be involved.

Our results indicate that the biochemical response is likely to be dependent on the presence of sstr$_2$. We also found a strong correlation between the presence of sstr$_2$ and symptomatic response. In pituitary somatotrophs, it has been shown that somatostatin may decrease growth hormone secretion through sstr$_2A$ and sstr$_2B$ by activating different G proteins (14). This may support the hypothesis that inhibition of hormone secretion by treatment with somatostatin analogues is not only dependent on the binding of an analogue to a receptor subtype but is also dependent on postreceptor signaling. It is obvious that patients with $[^{111}\text{In-DTPA-o-Phe}^1]$-octreotide uptake in tumors not expressing sstr$_2$ mRNA may have other subtypes that bind the molecule but fail to inhibit hormone secretion.

We have only focused on the biochemical and symptomatic responses in this study. However, during the last few years experimental data have shown that somatostatin analogues may play a role in growth control and induce apoptosis when administered at higher dose levels. It has been shown that sstr$_1$, sstr$_3$, and sstr$_5$ may inhibit cell proliferation, but through different intracellular pathways (15, 16). Whether this can be reproduced in patients still has to be investigated further.

In the future, new somatostatin analogues with specific binding to each one of the five sstr subtypes may be available for clinical use. Methods for identification of the different receptor subtypes will then be necessary. In situ hybridization may be such a method, and sstr scintigraphy may be used only to exclude patients lacking receptor expression from such treatment. Thus, by using in situ hybridization, detailed information concerning the subtypes of sstrs may be obtained, and this may be used to guide future therapy and help to select the analogues that should be used.

We conclude that in situ hybridization seems to be a reliable method for selecting carcinoid patients that may respond to somatostatin analogue treatment. The correlation to therapeutic outcome is
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