Quantitative evaluation of somatostatin receptor subtype 2 expression in sporadic colorectal tumor and in the corresponding

Quantitative Evaluation of Somatostatin Receptor Subtype 2 Expression in Sporadic Colorectal Tumor and in the Corresponding Normal Mucosa[1]

Claudia Casini Raggi, Antonino Calabrò, Daniela Renzi, Vittorio Briganti, Fabio Cianchi, Luca Messerini, Rosa Valanzano, Michela Cameron Smith, Camillo Cortesini, Francesco Tonelli, Mario Serio, Mario Maggi,[2] and Claudio Orlando

Clinical Biochemistry [C. C. R., C. O.], Gastroenterology [A. C., D. R.], Surgery [R. V., F. T.], Endocrinology [M. C. S., M. S.], and Andrology [M. M.] Units, Department of Clinical Physiopathology; Institute of Clinical General Surgery and Related Disciplines [F. C., C. C.]; and Institute of Anatomic Pathology [L. M.], University of Florence, Florence, and Department of Nuclear Medicine, Azienda Ospedaliera Careggi, [V. B.], Florence, Italy 50139

ABSTRACT

***Purpose*: The somatostatin (SS) receptor subtype 2 (sst2) is the principal mediator of the antiproliferative effects of SS and has the highest affinity for the commercially available SS analogues. The purpose of this study was to evaluate sst2 mRNA expression by quantitative reverse transcription-PCR (RT-PCR) in colon cancers and in corresponding normal tissues.**

***Experimental Design*: The expression of sst2 mRNA was measured with a quantitative method based on real time RT-PCR with TaqMan assay in 100 colon cancers and in the corresponding normal tissues. In a limited number of patients, these results were compared with those obtained by *in situ* hybridization ($n = 26$) and by *in vivo* imaging with ^{111}In-pentetreotide ($n = 17$).**

***Results*: Results obtained by quantitative RT-PCR on sst2 expression in colorectal cancer were significantly related to those obtained by *in situ* hybridization and ^{111}In-pentetreotide scintigraphy. Sst2 was expressed in all of the tumors investigated without any relationship with localization, grading, and stage of disease. Although the paired, unaffected mucosa tends to express a higher abundance of sst2 than the corresponding cancer samples, this difference did not reach a statistical significance. However, in patients with elevated carcinoembryonic antigen levels (>5 ng/ml) there was a significant loss of sst2 mRNA in the tumor when compared with its paired normal tissue.**

***Conclusions:* In this study we confirmed, by a quantitative method, that colorectal cancer does not express higher concentrations of sst2 mRNA than the corresponding unaffected tissue. Conversely, a loss of sst2 was found in patients with elevated preoperative concentrations of carcinoembryonic antigen, an unfavorable prognostic marker for colorectal cancer.**

INTRODUCTION

Colorectal cancer is one of the major causes of cancer-related mortality in the Western world. In Italy, 20,000–30,000 new cases of colorectal carcinoma are reported every year, with the highest prevalence in patients above 65 years of age (1). Advances made in the understanding of the disease, both in terms of clinical behavior and molecular pathogenesis, have been translated into improvements in its traditional therapy (2). Despite this, many patients continue to succumb to the disease. Therefore, it is important to identify new prognostic factors that may allow additional insight into the optimal treatment strategy for all patients. Although cancer stage is considered the most important independent factor for survival or recurrence after potentially curative surgery, many other independent factors have been identified (3). One of these may involve the characterization of SS[3] receptors status.

SS is a ubiquitous peptide involved in multiple cellular activities. In particular, SS regulates cell secretion and proliferation through a family of G-protein coupled receptor subtypes (ssts; Ref. 4). The antiproliferative effect of SS is determined in part indirectly through inhibition of the release of mitogenic hormones and growth factors, through inhibition of angiogenesis, and in part directly through ssts located on cell membranes (4).

Among the different sst subtypes identified recently (sst1-sst5), sst2 mediates the antiproliferative effect more efficiently than the others (5) and shows the highest affinity for SS analogue octreotide (6). SS receptors, particularly sst2, are commonly overexpressed in a wide variety of neoplasms, especially those arising from the neuroectoderm. Therefore, radiolabeled SS analogues are useful in the management of well-differentiated neuroendocrine malignancies such as carcinoid tumors (7). In addition, many recent studies showed that sst2 is often highly

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[2] To whom requests for reprints should be addressed, at Andrology Unit, Department of Clinical Physiopathology, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy. E-mail: m.maggi@dfc.unifi.it.

[3] The abbreviations used are: SS, somatostatin; sst2, somatostatin receptor subtype 2; RT-PCR, reverse transcription-PCR; CEA, carcinoembryonic antigen; pentetreotide, diethylenetriaminepentaacetic acid-D-Phe-1-octreotide; SPECT, single photon emission computed tomography; ROI, region of interest.

expressed not only by tumors of the neuroendocrine system but also by others, such as colorectal carcinoma (8–10).

Some recent studies have been performed to characterize the pattern of expression of the different sst mRNA subtypes in colon tumors, providing controversial results (11–13). Using conventional qualitative RT-PCR, Buscail *et al.* (11) found a heterogeneous expression of sst1-sst5 mRNA in colorectal carcinoma with a loss of sst2 mRNA expression in advanced stages. In another study, using both RT-PCR and *in situ* hybridization, Laws *et al.* (12) observed a retained expression of sst2 mRNA but a decreased expression of sst5 mRNA in late stage tumors. Vuaroqueaux *et al.* (13), using RT-PCR, showed that sst5, sst1, and sst2 mRNA subtypes were the most frequently expressed in a relatively large set of tumor and normal colon samples. Interestingly, loss of sst2 and sst5 mRNA expression in advanced stages was not demonstrated. A more recent study of the same group (14), using *in situ* hybridization, demonstrated that sst5 was by far the most expressed subtype in both normal and tumor colonic tissues.

The controversial results of these studies might be explained by the methods used. Indeed, both RT-PCR and *in situ* hybridization are not accurate enough in measuring *sst2* gene expression, and our previous experience in neuroblastoma clearly showed that only a quantitative determination of *sst2* gene expression was able to predict patient outcome. In fact, we demonstrated that in neuroblastoma, where the receptor is highly expressed, sst2 mRNA expression measurement not only gave relevant insights in terms of patient overall and disease-free survival but also represented the most relevant prognostic factor for this kind of tumor (15).

Similarly, we tested whether sst2 expression may possibly represent a prognostic factor for colon cancer. Furthermore, the exact determination of its expression might help to identify patients eligible for a new treatment modality based on SS analogues conjugated with cytotoxic agents or with radio-emitting molecules. Up to now, SS analogue therapy has been very disappointing in the management of advanced malignancy. Improvements in the treatment of solid tumors are then likely to come only from such therapies (16). A few studies showed that cytotoxic SS analogues containing doxorubicin or 2-pyrrolino-doxorubicin efficaciously inhibit growth of human breast and prostate cancers expressing sst2 and sst5 and, therefore, can be used for receptor-targeted chemotherapy in other sst-positive tumors such as colon cancer (17, 18). In addition, *in situ* radiotherapy with radiolabeled SS analogues has been successfully used for scintigraphic evaluation and management of patients with sst-positive neuroendocrine cancers (7, 19, 20).

The purpose of our study was to evaluate sst2 mRNA expression in a large number of surgically removed colorectal carcinomas by quantifying specific PCR products with an accurate quantitative RT-PCR method with TaqMan reaction (21). Moreover, sst2 mRNA expression was also quantified in paired normal tissues to evaluate the different expression of sst2 in tumor and normal colorectal tissues. However, quantitative RT-PCR does not overcome one of the main limits of the PCR procedures, which rely on the evaluation of a gene product derived from a mixture of nucleic acid from different cell populations. Therefore, in a limited number of patients, we compared results obtained by real time RT-PCR with those obtained by a semiquantitative *in situ* hybridization (15) performed on the same samples and also with those obtained *in vivo*, before surgery, by imaging with Octreoscan (22).

MATERIALS AND METHODS

Patients and Samples. Tissues were obtained from 103 patients with sporadic colorectal carcinoma (62 males and 41 females, age range: 46–89 years, mean = 66.8) scheduled for elective resection. In 17 unselected patients, SS receptor scintigraphy with ^{111}In-pentetreotide before surgery was also performed. Informed consent was obtained previously from all of the patients. For all of the patients at least one sample of both neoplastic and normal tissue (taken 10 cm apart from the neoplasm) were obtained. Samples were immediately snap frozen and stored in liquid nitrogen. Two patients were excluded from the study, because their tumors were identified as a lymphoma and as an epidermoid carcinoma. A third patient was excluded because of the absence of malignant neoplasia. Tumor was localized in the right colon in 34 patients, in the left colon (12 in the descending and 21 in the sigmoid colon) in 33 patients, and in the rectal portion in the remaining 33 patients. Histological examination was performed routinely in all of the cases. An adequate number of sections was sampled from each tumor. Slides were reviewed by the same pathologist without knowledge of SS receptor status. Tumor histotype and grade of differentiation were defined according to the WHO criteria (23). The pattern of cancer growth was assessed as expanding (when the tumor border was clearly demarcated) and as infiltrating (when cancer cells spread into the surrounding tissues without a distinct border; Ref. 24). All of the cases were staged according to the original Dukes' system. According to the histopathological grading, 5 tumors were G1, 61 were G2, 8 were G3, and 16 were colloid; the other four showed a mixed pattern of G2 plus colloid. Six were *in situ* tumors. CEA concentration, measured before surgery, was available for 92 patients.

Total RNA was extracted from each sample with RNeasy kit (Quiagen, Milan, Italy). Because *sst2* is an intron-less gene, each RNA sample was first submitted to a conventional PCR with the same primers and cycling for sst2 but without reverse transcription to exclude the presence of residual genomic DNA in the extracted specimens. Samples with residual DNA were treated with DNase until the disappearance of any DNA trace.

Quantitative Evaluation of sst2 mRNA Expression. The primers and probe for sst2 mRNA quantification to use with the ABI Prism 7700 Sequence Detection System were described elsewhere (21). Total RNA (400 ng) is reverse-transcribed according to recommended protocol. The PCR mixture contains primers (200 nM each) and 200 nM of the Taqman probe in a final volume of 25 μl. Amplification and detection were performed with the ABI Prism 7700 Sequence Detection System with the following profile: one step at 50°C for 2 min, one step at 95°C for 10 min, and 40 cycles at 95°C for 30 s and 60°C for 1 min. The amount of product was measured by interpolation from a standard curve with RNA extracted from neuroblastoma cell line CHP404, which overexpresses sst2 mRNA. CHP404 RNA (1 μg) was reverse transcribed, and cDNA was then serially diluted to obtain five standard solutions to be used in the PCR reaction to generate the reference curve (21).

sst2 Scintigraphy. Pentetreotide and ^{111}In-chloride were obtained from Mallinckrodt Medical BV. Radiolabeling was performed according to the instructions of the manufacturer. Patients were injected with 5 MBq/kg of ^{111}In-pentetreotide i.v. without any previous preparation. After the injection (3 h) a planar whole-body acquisition was performed, with preset-counts modality and with a 128 × 128 matrix, zoom 1.0, and high-resolution collimator (only 172 keV peak). After the injection (4 h) a tomographic acquisition was performed on the suspected region of the body with a 64 × 64 matrix, zoom 1.0, high-resolution collimator (only 172 keV peak), and 40 frames for head of 454 s (three-head camera). After the administration of ^{111}In-pentetreotide (24 h), SPECT acquisition was then repeated with the same parameters on the same body region (22). A tomographic reconstruction by filtered backprojection was performed with convolution low-bass filter (cutoff 0.25 cycles/pixel and filter order 4.0) for every tomographic registration (22).

A normal ROI on the suspected area (ROI_T) was drawn on transaxial slice of the SPECT at 4 h; the same ROI was repositioned on the same area of the following SPECT at 24 h. The ROI was also repositioned on the normal contralateral tissue (ROI_{NT}) for every transaxial slice of the SPECTs. Then a ratio between ROI_T and the ROI_{NT} was calculated for every transaxial SPECT slice. According to the SPECT time, ROI_T:ROI_{NT} ratios were evaluated at 4 h [(ROI_T:ROI_{NT})4 h] and 24 h [(ROI_T:ROI_{NT})24 h]. If the activity in ROI_T is attributable to overexpression of sst2, the ROI_T:ROI_{NT} ratio should increase between the SPECT at 4 and 24 h. Conversely, if the activity in ROI_T at 4 h is attributable to "nonspecific" uptake, the ROI_T:ROI_{NT} ratio should be stable or decrease between 4 and 24 h (22). The increase (ROI_T:ROI_{NT} INC) of the ROI_T:ROI_{NT} ratio is expressed by:

$$ROI_T{:}ROI_{NT}\ \text{INC} = [(ROI_T{:}ROI_{NT})24\ \text{h} - (ROI_T{:}ROI_{NT})4\ \text{h}]/(ROI_T{:}ROI_{NT})4\ \text{h}.$$

***In Situ* Hybridization.** Frozen sections (7-μm thick) from both tumor and macroscopically uninvolved tissue samples were collected onto gelatin/chrome alum-coated slides, dried briefly on a hot plate at 80°C, and fixed in 4% paraformaldehyde/PBS, (pH 7.4) for 20 min. After three washes in PBS and short air drying, sections were immediately used for *in situ* hybridization. For the preparation of the RNA probe for sst2, the 284-bp fragment of the human sst2 cDNA obtained by RT-PCR was subcloned into the appropriate restriction site of the plasmid pGEM-T Vector (Promega, Madison, WI), as described previously (25, 26). Transcription and labeling of RNA probes were performed using 60 μCi of [^{35}S]-uridine-5′-(α-thio)-triphosphate (1250 Ci/mmol; New England Nuclear, Dreieich, Germany). The specific activity routinely obtained was 1.2–1.4 × 10^9 cpm/μg. RNA probes were stored at −80°C and used within 2 weeks. Prehybridization, hybridization, removal of nonspecifically bound probe by RNase A digestion, and additional washing procedures were performed for positive- and negative-strand RNA probes as described elsewhere (25).

Quantitative evaluation of sst2 mRNA expression was performed by two independent observers who did not know the results of real time RT-PCR with the aid of a computerized video image analysis system (Quantimet Q500 MC; Leica Cambridge Ltd., Cambridge, England). For the quantitation of *in situ* hybridization results, six visual fields were chosen randomly from each section and analyzed under a dark field microscope equipped with a ×20 lens. The autoradiographic signal corresponding to the specific hybridization was acquired by a CCD video camera connected to the microscope, converted to digital, and transformed into pixel units. The threshold of specific detection was automatically calibrated on control sections hybridized with the corresponding sense probe. The results, evaluated in terms of percentage of the total area occupied by the sst2 autoradiographic signal, were expressed as mean ± SD (15).

Statistical Analysis. Statistical analyses were performed with software from SPSS, Inc. (Chicago, IL). Significance of the differences was evaluated by Student's *t* tests for paired or independent samples, or by one-way ANOVA, as indicated. In matched tumor (T)-normal (N) pairs, the ratio between sst2 expression values (T:N) was considered increased (*i.e.*, tumor more than normal) when T:N > 1.2 and decreased when T:N < 0.8 (tumor less than normal).

RESULTS

Semiquantitative *in Situ* Hybridization. Twenty-six tumors were analyzed by *in situ* hybridization. Specific *sst2* gene expression was observed in all of the malignant samples as well as in the corresponding unaffected tissues examined ($n = 22$).

Fig. 1 shows sst2 mRNA expression in representative sections of colon cancer and in the paired uninvolved mucosa. In the normal colon, *sst2* gene expression was predominantly localized in the epithelial cells lining the mucosal surface and crypts. Weak autoradiographic labeling was also observed on a few lamina propria mesenchymal cells. Smooth muscle cells of the muscularis mucosae and propria, and the muscular wall of larger submucosal blood vessels, were also positive. Of note, a prominent autoradiographic signal was sometimes found on inflammatory cells of the normal mucosa adjacent to well-differentiated neoplasms (Fig. 1, *a* and *b*).

In tumor samples, a relatively homogeneous autoradiographic signal was found throughout all of the malignant cells (Fig. 1, *c–f*). Specific labeling was also noted on some cells sparsely distributed in the peritumoral stroma which, on the basis of their morphology, were tentatively identified as inflammatory cells (*e.g.*, lymphocytes and macrophages) and fibroblasts. In addition, endothelial cells of capillaries and venules displayed low but still detectable sst2 mRNA expression. Lending support to the specificity of these results, control sections hybridized with the sense (anticomplementary, negative control) sst2 probe displayed only a limited number of autoradiographic grains, which cannot be distinguished from the background labeling (Fig. 1, *g* and *h*).

The semiquantitative evaluation of the positive autoradiographic signals indicated that sst2 expression was quite variable in the 26 tumors examined (2.9–16.4% total area with a mean value of 9.12 ± 3.91).

Semiquantitative *in Vivo* sst2 Scintigraphy. We found ligand binding in all of the 17 patients investigated with the semiquantitative ^{111}In-pentetreotide sst2 scintigraphy. In 13 of them we found a specific temporal increase in ROI_T:ROI_{NT}

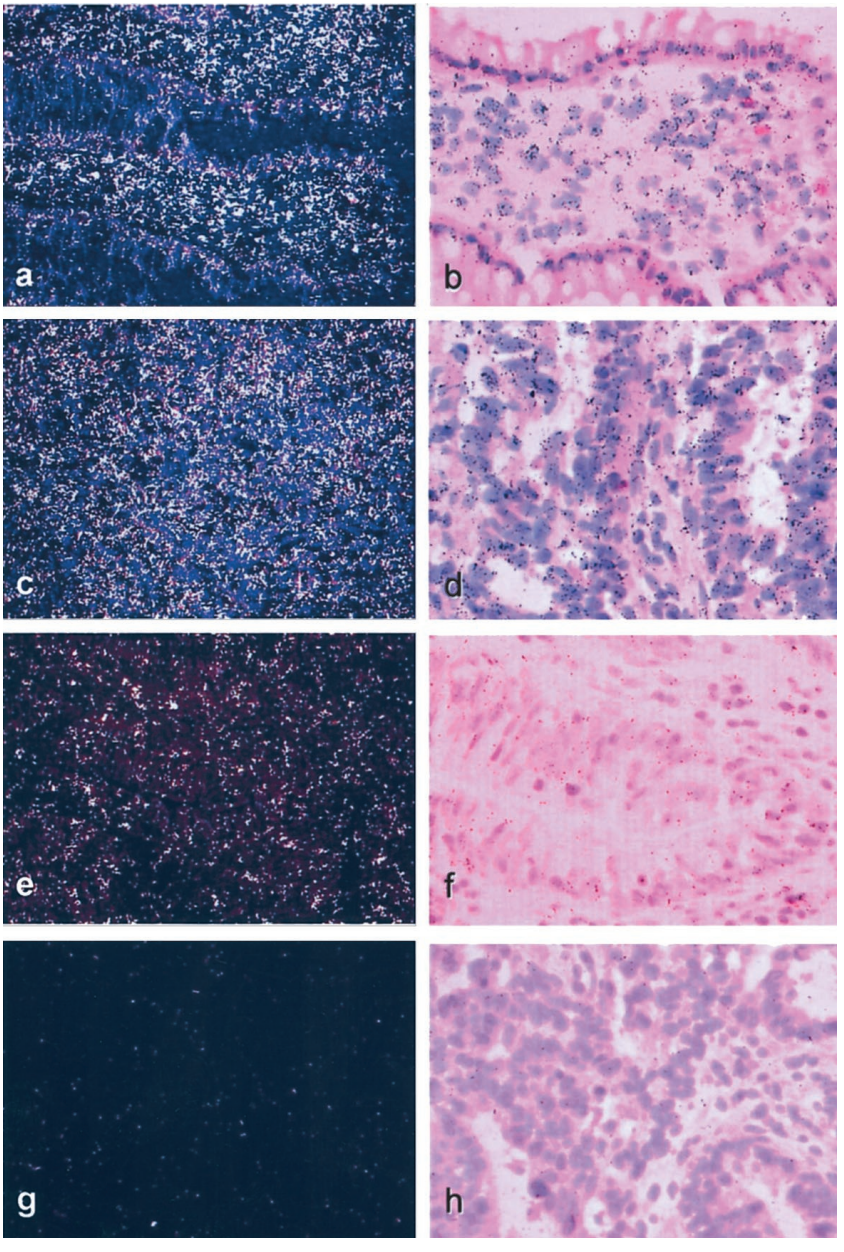

Fig. 1 Dark- and bright-field photomicrographs showing *in situ* hybridization to sst2 mRNA with antisense (*a–f*) and sense (*g* and *h*) ^{35}S-labeled RNA probes. Intense sst2 mRNA expression is evident in the normal mucosa adjacent to a well-differentiated cancer of the left colon (*a*); a bright-field photomicrograph at greater magnification clearly shows sst2 mRNA expression in inflammatory cell of the lamina propria (*b*); strong sst2 expression is also noted in malignant cells from the corresponding tumor (*c* and *d*), whereas very low amounts of sst2 RNA transcripts are evident in a representative section from a cancer of the right colon (*e* and *f*); a control section hybridized with the sense anticomplementary probe shows only nonspecific background signal (*g* and *h*). Exposure time: 6 weeks. Original magnification: *a, c, e,* and *g,* ×130; *b, d, f,* and *h,* ×260.

ratio (mean increase: 78.92 ± 23.11%; range 3–295%). Among these patients the increase was <45% in 6 of 13 subjects and >45% in 7 of 13 subjects. In the other 4 patients the increase in ROI_T:ROI_{NT} ratio was absent. Fig. 2 shows representative SPECT in two patients who were classified, on the basis of this method, as positive and negative for the presence of SS-binding sites, respectively.

Quantitative RT-PCR. All of the tumor tissues analyzed with real time RT-PCR showed *sst2* gene expression. Nevertheless, the level of this expression was quite variable, ranging from 7.9×10^5 to 1.2×10^9 molecules/μg of total RNA, with a mean value of $10.3 \pm 1.9 \times 10^7$. Results obtained in colorectal carcinoma with real time RT-PCR were significantly related with those derived from the two aforementioned techniques. Thus, we found significantly positive relationships among measurements of sst2 expression obtained by real time RT-PCR and *in situ* hybridization ($r = 0.51$; $P < 0.01$; $n = 26$) or by real time RT-PCR and *in vivo* imaging with ^{111}In-pentetreotide ($r = 0.51$; $P < 0.05$; $n = 17$).

Relationship between sst2 Expression in Colorectal Carcinoma and Clinical-Pathological Features. Table 1 shows the mean values (± SE) of sst2 mRNA expression in colorectal carcinoma as evaluated by either *in situ* hybridization ($n = 26$) or RT-PCR ($n = 100$), as a function of the most common clinical-pathological features for colorectal carcinoma. One-way ANOVA did not find statistically significant associations between sst2 expression and any of the clinical-pathological parameters investigated. It is interesting to note that patients

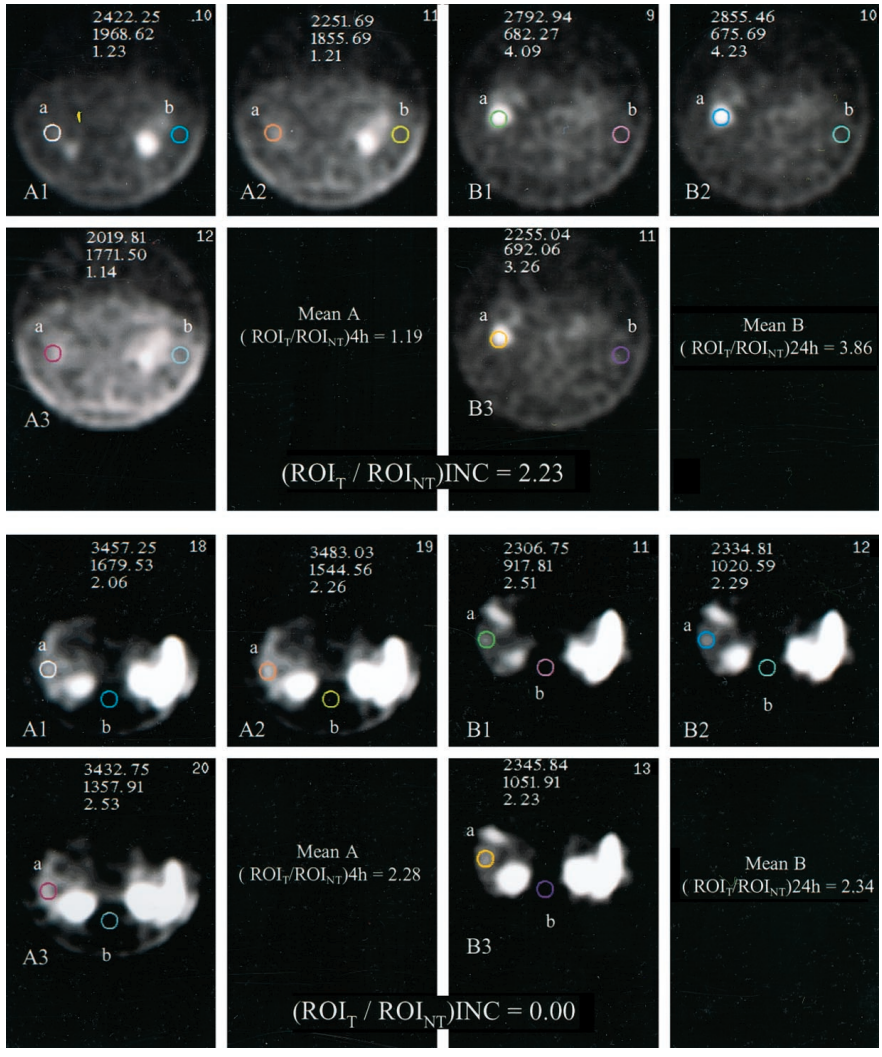

Fig. 2 Typical SPECT studies in two patients with colorectal cancer. After ^{111}In-pentetreotide injection, ROIs were extracted at two different times in three consecutive slices (*A*, after 4 h, first three boxes; *B*, after 24 h, last three boxes) in pathological (ROI_T, *circles a*) and normal (ROI_{NT}, *circles b*) tissues. The ratio ROI_T:ROI_{NT} was then calculated. The first number at the top of each box represent counts/pixels in pathological tissue, while the second one represents counts/pixels in the selected normal tissue. Below these numbers, the individual ratio between values is also reported. Calculated mean ratio ROI_T:ROI_{NT} is shown in an additional box at 4 h (*Mean A*) and 24 h (*Mean B*) in each panel. *Top panel*, patient with a specific increase between 4 and 24 h in ROI_T:ROI_{NT}. *Bottom panel*, patient without increase between 4 and 24 h in ROI_T:ROI_{NT}. The increase (ROI_T:ROI_{NT} INC) of the ROI_T:ROI_{NT} ratio is expressed by ROI_T:ROI_{NT} INC = [(ROI_T:ROI_{NT})24 h − (ROI_T:ROI_{NT})4 h]/(ROI_T:ROI_{NT})4 h.

with an elevated value of preoperative CEA (>5 ng/ml) express a lower concentration of sst2 mRNA than patients with normal CEA levels, even if this difference is not statistically significant ($P = 0.06$ for *in situ* hybridization and $P = 0.13$ for RT-PCR).

Relationship between sst2 Expression in Colorectal Carcinoma and the Corresponding Unaffected Colorectal Samples. *sst2* gene expression was also evaluated in the adjacent unaffected colorectal mucosa by both real time RT-PCR ($n = 100$) and semiquantitative *in situ* hybridization ($n = 22$). Even in these normal colorectal samples, the expression of sst2 showed a large variability when analyzed either by real time RT-PCR (2.9×10^5-2.8×10^9 molecules/μg total RNA, with a mean value of $16 \pm 3.8 \times 10^7$) or by semiquantitative *in situ* hybridization (4.46–16.49% total area, with a mean value of 9.0 ± 0.6). Table 1 also shows the results obtained by real time RT-PCR in the unaffected colorectal tissue as well as in the corresponding cancer samples for comparison. The matched tumor-normal pairs showed a higher *sst2* gene expression in the tumor than in the corresponding normal tissues in 35 subjects (35%; T:N > 1.2), whereas in 56 patients we found the opposite (56%; T:N < 0.8). In 9 patients, the levels of sst2 expression in the normal and pathological tissues were rather similar ($0.8 \leq$ T:N ≤ 1.2). We did not find any statistically significant difference in sst2 expression between normal and tumor samples, although malignant tissue tends to express a lower abundance of sst2 than the normal mucosa. A lack of significant difference was also found when normal and pathological tissues were compared according to sex, age, localization, stage, and grading of disease. However, as reported in Table 1, patients with elevated concentrations of CEA (>5 ng/ml) had a significantly lower sst2 mRNA expression in tumors ($5.7 \pm 1.74 \times 10^7$ molecules/μg of total RNA) when compared with the corresponding normal tissue ($19.6 \pm 6.29 \times 10^7$ molecules/μg of total RNA; $P = 0.028$). Such a difference was not found in patients with normal concentrations of CEA (<5 ng/ml; $11.9 \pm 2.83 \times 10^7$ *versus* $15.8 \pm 5.27 \times 10^7$ molecules/μg of total RNA; $P = 0.509$).

We did not find any significant difference when matched tumor-normal pairs of colorectal carcinoma were evaluated by semiquantitative *in situ* hybridization (not shown). The ratio

Table 1 sst2 mRNA expression in colorectal tumor and corresponding normal tissues, measured by *in situ* hybridization by quantitative RT-PCR

	In situ hybridization in neoplastic tissue				Real time RT-PCR							
					Neoplastic tissue				Normal tissue			Normal *vs* neoplastic
	n	Mean	SE	One-way Anova	*n*	Mean	SE	One-way Anova	Mean	SE	One-way Anova	Student's *t* test
Total	26	9.12[a]	3.91		100	1.0×10^{8}[b]	1.9×10^{7}		1.6×10^{8}[b]	3.8×10^{7}		$P = 0.17$[c]
Age												
<55	2	10.35	3.75	$P = 0.79$	8	9.2×10^{7}	5.4×10^{7}	$P = 0.84$	2.5×10^{8}	1.6×10^{8}	$P = 0.56$	$P = 0.20$
>55	24	9.09	0.80		92	1.0×10^{8}	2.1×10^{7}		1.5×10^{8}	3.9×10^{7}		$P = 0.28$
Sex												
Males	16	8.89	1.07	$P = 0.61$	60	1.1×10^{8}	2.6×10^{7}	$P = 0.56$	2.1×10^{8}	6.1×10^{7}	$P = 0.13$	$P = 0.15$
Females	10	9.67	1.08		40	9.0×10^{7}	3.0×10^{7}		9.0×10^{7}	1.8×10^{7}		$P = 0.98$
Localization												
Right	10	8.73	1.36	$P = 0.88$	34	5.7×10^{7}	1.3×10^{7}	$P = 0.10$	1.7×10^{8}	8.3×10^{7}		$P = 0.18$
Left	10	9.66	1.27		32	1.6×10^{8}	4.8×10^{7}		1.7×10^{8}	6.3×10^{7}	$P = 0.96$	$P = 0.95$
Rectum	6	9.18	1.46		34	9.86×10^{7}	3.2×10^{7}		1.5×10^{8}	4.4×10^{7}		$P = 0.29$
Dukes' stage												
A	6	9.50	1.59	$P = 0.99$	14	7.0×10^{7}	3.3×10^{7}	$P = 0.54$	1.6×10^{8}	9.3×10^{7}	$P = 0.81$	$P = 0.19$
B	7	8.77	1.47		46	1.3×10^{8}	3.5×10^{7}		1.7×10^{8}	4.7×10^{7}		$P = 0.53$
C	7	9.30	1.39		31	8.2×10^{7}	3.0×10^{7}		1.8×10^{8}	9.0×10^{7}		$P = 0.33$
D	6	9.25	2.10		9	7.0×10^{7}	4.2×10^{7}		4.3×10^{7}	1.8×10^{7}		$P = 0.60$
Grading												
G0/G1	5	9.92	0.88	$P = 0.17$	11	2.2×10^{7}	9.5×10^{6}	$P = 0.28$	5.9×10^{7}	2.4×10^{6}		$P = 0.15$
G2/G3/mix	17	9.59	1.02		74	1.2×10^{8}	2.5×10^{7}		1.9×10^{8}	5.0×10^{7}	$P = 0.30$	$P = 0.18$
Colloids	4	6.57	2.11		15	7.2×10^{7}	3.8×10^{7}		6.2×10^{7}	1.9×10^{7}		$P = 0.69$
Pattern												
Infiltrating	11	8.64	1.34	$P = 0.57$	57	1.08×10^{8}	2.33×10^{7}	$P = 0.93$	1.6×10^{8}	5.8×10^{7}	$P = 0.67$	$P = 0.42$
Pushing	12	9.63	1.12		36	1.12×10^{8}	4.06×10^{7}		1.9×10^{8}	5.0×10^{7}		$P = 0.18$
CEA												
<5 ng/ml	16	10.17	0.84	$P = 0.06$	60	1.2×10^{8}	2.8×10^{7}	$P = 0.13$	1.6×10^{8}	5.3×10^{7}	$P = 0.64$	$P = 0.51$
>5 ng/ml	9	6.92	1.34		32	5.7×10^{7}	1.7×10^{7}		2.0×10^{8}	6.3×10^{7}		$P = 0.03$

[a] Percentage of total area.
[b] Molecules of sst2 mRNA/μg total RNA.
[c] *t* test for paired samples.

between tumor and normal (T:N) values was increased in 45.5%, decreased in 40.9%, and similar in 13.6%.

DISCUSSION

In this study we reported the largest series of colorectal carcinoma thus far investigated for the evaluation of the expression of sst2, which binds with the highest affinity clinically available SS analogues.

In recent years, several laboratories provided evidence for the expression of SS receptors in colorectal carcinoma. Such evidence was mainly based on radioligand-binding procedures performed on membranes (8, 27) or tissue slices (28) from limited series of primary colorectal carcinomas or from colorectal carcinoma cell lines. Studies *in vitro* on colorectal carcinoma cell lines (29–32) or *in vivo* in nude mice bearing colorectal carcinoma cell xenografts (29, 33) indicated that treatment with SS or its analogues greatly decreased growth of several but not all of the colorectal carcinoma cell lines. Indeed, no effect of SS was observed in SS receptor-negative cell lines (34), indicating that the antiproliferative effect of SS in colorectal carcinoma cells was mediated by the expression of specific receptors (35). In the meantime, SS analogues labeled with ^{123}I or ^{111}In for the *in vivo* imaging of SS-binding sites have been developed and used in nuclear medicine to visualize receptor-positive tumors, including colorectal carcinoma and their metastases (36). Results from these studies prompted several clinical trials to test the relative efficacy of SS analogue treatment in patients with colorectal carcinoma (37–42). In a subset of patients, a short-term octreotide therapy decreased markers of cell proliferation in the primary tumor (38, 40). However, in the majority of trials, SS analogue therapy has been very disappointing in terms of survival or disease stabilization (37, 41, 42). Only in a relatively recent study a significant advantage in terms of survival was reported (39). These contradictory findings might be explained by the different dose of SS analogues given to patients in the different trials or, most probably, by the inappropriate selection of patients (advanced colorectal carcinoma). Indeed, advanced colorectal carcinoma might express less sst2 receptors than early stage tumors. In fact, some (11) but not all (12, 13) of the previous studies indicated a loss of sst2 in the most advanced stages of the disease. Because sst2 receptor expression has not been studied in any of these clinical trials, it is possible that colorectal cancer patients with a low concentration of sst2 were also treated with SS analogues (11). In addition, it is possible that the antineoplastic activity of SS analogues does not produce an adequate clinical response as adjuvant therapy to surgery or

chemotherapy, even in patients expressing a relative abundance of sst2. However, it is important to understand whether tumor sst2 expression is higher or at least not lower than the expression of unaffected mucosa. Hence, the present study is important for the following reasons: (*a*) it provides a quantitative estimation of sst2 expression in a rather large cohort of colorectal carcinomas; (*b*) it reports the simultaneous determination of sst2 in tumor as well as in the neighboring normal mucosa; and (*c*) it uses three different methods to evaluate sst2 expression.

We essentially found that all of the 100 colorectal carcinoma tumors expressed measurable amounts of sst2 mRNA when analyzed by real time RT-PCR, with a mean value of expression (1.03×10^8 molecules/μg of total RNA) not so different from the mean value (9.8×10^8 molecules/μg of total RNA) reported by our group in neuroblastoma, a pediatric neuroendocrine tumor (15). Accordingly, a positive signal for *sst2* gene was detected by *in situ* hybridization in all of the tumors, although the size of the sample analyzed was limited ($n = 26$). It is important to note that quantitative results on sst2 mRNA as derived by RT-PCR and by *in situ* hybridization evaluation are significantly related. This result is in perfect agreement with a previous report on neuroblastoma (15). An important finding of the *in situ* hybridization study was that the autoradiographic labeling was not only present in tumor cells but also on blood vessels, inflammatory cells, and even in the epithelial and mesenchymal cells of the surrounding unaffected mucosa. Accordingly, we did not find any significant difference in sst2 expression when matched tumor-normal pairs were analyzed by either quantitative RT-PCR or *in situ* hybridization, although, on average, tumor samples expressed a lower amount of sst2 mRNA than the unaffected samples. In fact, about half of the tumor samples showed a lower expression of sst2 mRNA than the corresponding unaffected tissues (RT-PCR or *in situ* hybridization). In line with this observation are the results of the preoperative *in vivo* imaging of colorectal carcinoma patients with ^{111}In-pentetreotide performed in a small subgroup of these patients. Semiquantitative evaluation of ^{111}In-pentetreotide uptake indicates a high increase in the ROI_T:ROI_{NT} ratio (>45%) and, therefore, a high density of octreotide binding sites (34) in only a fraction of patients (7 of 17). Interestingly, we found a significant positive correlation between quantitative results of ^{111}In-pentetreotide scintigraphy and RT-PCR, as reported previously in an other study (22). These findings indicate that only a minority of colorectal carcinomas shows an increased expression of *sst2* gene and protein in tumors when compared with the corresponding normal tissue. Therefore, only a minority of colorectal carcinoma might be successfully targeted with SS analogues for diagnostic or therapeutic purposes. Our results are in agreement with several previous observations showing, in more limited series, a low abundance of sst2 in colorectal carcinoma (8, 27, 28).

Analysis of the distribution of *sst2* gene expression in colorectal carcinoma as a function of several clinical-pathological features did not show any statistically significant relationship between tumor sst2 expression and any of the parameters examined, including localization, grading, or stage of the disease. The same results were obtained when matched tumor-normal colorectal carcinoma pairs were analyzed. Hence, we cannot confirm that there is a loss of sst2 expression in more advanced stages of the disease (11). However, we found that patients with an elevated preoperative CEA (>5 ng/ml) showed a significantly lower expression of sst2 (three-fold) in the tumor than in the corresponding unaffected tissue. The *CEA* gene is one of the most widely expressed genes in colorectal carcinoma cells. CEA protein sheds in elevated amounts in peripheral circulation in about half of the patients with colorectal carcinoma (43). Preoperative measurement of CEA protein concentration is, up to now, the only serum marker recommended for staging and surgical treatment planning in colorectal carcinoma (44, 45). Postoperative serum CEA protein measurement is also the most effective approach to follow the course of colorectal carcinoma (46, 47). In addition, an elevated preoperative CEA level is generally accepted as a poor prognostic indicator (45, 48). The finding that *sst2* gene expression is markedly reduced in tumor samples of patients with elevated CEA concentrations suggests that *sst2* gene expression in colorectal carcinoma might be related to a favorable outcome, as we reported previously for the neuroendocrine tumor neuroblastoma. However, the prognostic relevance of sst2 determination in colorectal carcinoma should still be demonstrated in long-term follow-up of patients.

In conclusion, our study, based on a quantitative PCR approach, seems to give a direct confirmation to previous qualitative findings (11), demonstrating that colorectal carcinoma does not express a high abundance of sst2 but tends to express a lower receptor concentration when compared to the corresponding unaffected tissue. Loss of sst2 seems to be a relevant event in patients with elevated preoperative concentration of CEA, a poor prognostic indicator for colorectal carcinoma.

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