Quantitation of Somatostatin Receptor Type 2 Gene Expression in Neuroblastoma Cell Lines and Primary Tumors Using Competitive Reverse Transcription-Polymerase Chain Reaction


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ABSTRACT

We previously reported the presence of somatostatin (SS-14)-binding sites in a wide panel of human neuroblastoma (NB) tumor cell lines. Given that the adrenal gland and its relative embryonal and adult tumors express an abundance of mRNA for somatostatin receptor type 2 (sst2) mRNA, we studied the quantitative expression of sst2 in 6 NB cell lines and 15 primary tumors using competitive reverse transcription (RT)-PCR. This method uses an insertion mutant of the target gene as a competitor for the RT-PCR reaction, thus allowing exact quantitation of sst2 mRNA abundance. We found expression of specific transcripts for sst2 in all of the NB cell lines and tumors investigated (range, 9 x 10^2-4 x 10^9 molecules/μg RNA). In NB cells, the expression of sst2 was highly correlated with SS-14-binding sites (R = 0.93). In primary tumors, sst2 was positively related to the expression of the neuroendocrine marker secretogranin II (P < 0.05) and negatively related to N-myc amplification (a poor prognostic factor, P < 0.005) and metastatic dissemination (P < 0.05). In addition, Kaplan-Meier curves indicate that sst2 expression is positively related to survival (P = 0.01). In a patient with stage IVs disease (a spontaneously regressing form), we found the highest sst2 expression (4 x 10^9 molecules/μg RNA), a value relatively similar to that of normal adrenal. In conclusion, these data indicate that quantitation of sst2, as assessed with competitive RT-PCR, could represent a new prognostic tool in the neuroendocrine tumor NB. Since sst2 recognizes octreotide with high affinity, these findings could also have both diagnostic and therapeutic value.

INTRODUCTION

The recent cloning of at least five different isoforms of SS receptors (sst1-sst5) confirmed previous evidence on SS receptor heterogeneity (1, 2). Indeed, the presence of different subtypes of SS receptors was hypothesized early on, on the bases of different biological responsiveness to SS-14 and SS-28; it was later confirmed by the use of selective pharmacological probes (3). Of these, shortened octapeptide analogues of SS have been widely used to characterize SS isoreceptors. In particular, it was noted that just a subset of SS receptors recognizes octapeptide analogues with high affinity (4, 5). One of these octapeptide analogues, octreotide, has recently become commercially available in Europe and the United States for the treatment of endocrine and gastrointestinal diseases. In addition, ^123^I- or ^111^In-octreotide derivatives have been extensively used for in vivo imaging of SS receptors (6, 7). Combined in vitro radioligand-binding and in vivo scintigraphic studies indicate that SS receptors are not only localized in gastrointestinal and endocrine tissues but are also present in several neoplastic tissues (8). Hence, ^111^In-DTPA-D-Phe\(^3\)octreotide has been successfully used to visualize receptor-positive tumors and their metastases. The relative relevance of SS receptors in cancer has been further raised by the observation of an antiproliferative activity by SS and analogues (8, 9) as well as by the recent demonstration of active internalization of ^125^I-Tyr\(^3\)octreotide by pituitary tumor cells in vitro (10). Thus, SS receptor-positive tumors could either be treated with unlabeled SS analogues or targeted by α- or β-emitting isotopes coupled with these analogues.

Among the different SS receptor subtypes so far identified, sst2 binds octreotide with the highest affinity (1) and is thought to mediate the antiproliferative effect of octapeptide analogues through the stimulation of tyrosine phosphatase activity (11, 12). The subtype sst2 of SS receptors is widely distributed in the central nervous system, whereas in the periphery is the most abundant subtype in the rat (1) and human (13) adrenal gland. Accordingly, tumors in adult and embryonal adrenal medulla, such as pheochromocytoma (13, 14) and NB (9), express a relative abundance of sst2 mRNA (15).

We previously reported the presence of SS-14-binding sites in a wide panel of human NB tumor cell lines (16). However, only in a subset (30%) did we find high-affinity binding sites mediating biological functions. These included inhibition of adenylate cyclase activity, modulation of intracellular calcium levels, and inhibition of cell proliferation through a relative

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3 The abbreviations used are: SS, somatostatin; sst2, somatostatin receptor type 2; NB, neuroblastoma; RT, reverse transcription.
accumulation of cells in G0-G1 (16). Since rather identical biological activity has been observed in stable, transfected cells expressing sst2, we hypothesized the presence of sst2 in human NB cells. We therefore initiated studies to quantitate the expression of sst2 mRNA in human NB cells. In addition, we measured the expression of sst2 mRNA in tissue samples from patients affected by NB at different stages of the disease. The presence of sst2 transcripts in human tumors had previously been evaluated using conventional qualitative or semiquantitative methods such as Northern blot analysis (17), in situ hybridization (15), or RT-PCR (13, 14, 18). To date, the latter is the most powerful for the detection of the low abundant species of mRNAs in small samples of total RNA. However, quantitation of PCR products could be cumbersome, even in the presence of an internal standard (i.e., housekeeping genes) which requires a different pair of primers than the target gene. We therefore developed a method that uses an in vitro synthesized insertion mutant of the same sst2 mRNA as competitor for the RT-PCR reaction. Since the mutant and wild-type sst2 share the same primer recognition sites and are simultaneously subjected to RT and amplification, any variable affecting any single one of these steps has the same effect on both, even after extensive PCR cycling. Size differences between target and competitor RNA allow easy identification and quantitation on ethidium bromide-stained gels.

MATERIALS AND METHODS

Tumor and Tissue Samples. Fifteen primary NBs were collected for intraoperative diagnosis at the Department of Pathology, Regina Margherita Hospital (Turin, Italy). Two adjacent blocks were isolated: the first was fixed in 10% formalin for conventional histology and the second was immediately snap frozen in liquid nitrogen for mRNA and DNA extraction and analysis. Tumor grading and stage were determined in H&E-stained sections according to Shimada et al. (19) and Evans et al. (20), respectively. Adrenal samples were obtained at the time of surgery from one patient undergoing unilateral nephrectomy for renal carcinoma. A testicular specimen was obtained from a patient, complaining of infertility, who was being investigated for suspected obstructive azoospermia.

Total RNA and DNA were simultaneously extracted using the guanidine thiocyanate-cesium chloride method (21). The DNA bands obtained after centrifugation in a CsCl density gradient were freed from salts using dialysis. Northern blot analysis for chromogranin expression was performed as previously reported (22). Results were quantitatively assessed using laser densitometry and expressed as arbitrary units of absorbance (22).

Cell Lines. The following human neuroblastoma cell lines were studied: SK-N-AS (Dr. L. Nelson, ICI Americas, Wilmington, DE; Refs. 23 and 24); CHP-404, CHP-134B (Dr. A. Evans, Children's Hospital of Philadelphia, Philadelphia, PA; Refs. 25 and 26); SMS-KCN (Dr. Raymonds, UCLA, Los Angeles, CA; Ref. 27); SH-SY5Y (28); and LA-N-5 (Dr. Seeger, UCLA; Ref. 29). All cell lines were grown in RPMI 1640 supplemented with l-glutamine (2 mM), penicillin (50 units/ml), streptomycin (50 μg/ml), and 10% FCS. Total RNA was extracted using a standard phenol-chloroform procedure as described previously (30).

Construction and Cloning of Competitor Template for RT-PCR. For RNA competitor construction, two primers of primers were synthesized. External primers sst2/R and sst2/L were the same as those used for PCR amplification of cDNAs, the former being complementary to the RNA and thus used also for RT priming of cDNA. A second pair of internal primers (sst2/+1 and sst2/+2) contains, at the 3' end, a sequence of 20 nucleotides complementary to contiguous DNA sequences on opposite strands of sst2 cDNA and two sequences of 36 nucleotides, at their 5' ends, unrelated to the target and complementary to each other. These sequences allowed the annealing of the two internal primers and the construction of a competitor in a two-step recombinant PCR.

The procedure for competitor construction is reported in Fig. 1. Competitor plasmid pSSR-I was prepared using a modification of the overlap extension method as detailed previously (31, 32). Then sst2 mRNA from the CHP-404 cell line was reverse transcribed and amplified with external primers. Amplified cDNA was submitted to two separate PCR reactions combining primers sst2/R with sst2/+1 and sst2/L with sst2/+2. The two products of amplification were resolved on a 12% polyacrylamide gel, stained with ethidium bromide, eluted from the gel, mixed, and denatured in the thermal cycler at 95°C for 5 min in a 25-μl solution containing 1.5 mM MgCl2, 2.5 μl 10× PCR reaction buffer (Perkin Elmer-Cetus, Norwalk, CT), 1.25 units Taq polymerase, and 0.25 mM each deoxyribonucleotide triphosphate. The complementary tails of the two PCR products were then annealed in a single PCR cycle. The mixture remained for 60 s at 94°C, then reached 65°C in 10 min, and the annealing was completed at 65°C for 120 s. PCR extension was performed at 72°C for 7 min. This preparation was submitted to PCR in a final volume of 50 μl with external primers sst2/R and sst2/L to obtain a 320-bp fragment. The product of this PCR was cloned on a 12% polyacrylamide gel. After the elution from the gel, this fragment was cloned in the cloning site of the pGEM-T vector system (Promega, Madison, WI) downstream to the T7 RNA polymerase promoter (Fig. 1). The nucleotide sequence of the insert in plasmid pSSR-I was determined with the dyeoxysequencing method using primers in the regions flanking the vector polylinker.

Large amounts of competitor RNA were obtained by in vitro run off transcription with T7 RNA polymerase using a Ribo-Max kit (Promega) from 50 μg of pSSR-I after linearization with Sall. Template DNA was removed by DNase I digestion, and RNA competitor was quantified by spectrophotometric reading. Competitor concentration was expressed in terms of molecules/μl. The sequences of external and internal primers were: sst2/L (402-421), 5'-CAGTCTAGCAGCATCGACCGGCA-3' (17); sst2/R (665-684), 5'-GCAAAGACAGATGATCIG- GTGA-3'; sst2/+1(556-575), 5'-GCACTAIGATCCICGC- AGCAIATAICA-3'; sst2/+2(536-555), 5'-GATGCGIATCICGC- GACIIGAIGACAGATCCAGACCACTGGGAGGAGAG-3'; and sst2/+2(536-555), 5'-GATGCGTATCCTGC- GAGTTCGAIGACATCCCTGCAGTTCGCCGAGGAGAAG-3'.

Competitive RT-PCR for SSTR-2 mRNA Measurement. One μg of each RNA sample was reverse transcribed with 1 μl of 15 mM antisense primer (SST2/R) along with 1 μl
Fig. 1. Construction and cloning of competitor template for competitive RT-PCR determination of sst2 gene expression. A sst2 cDNA was obtained by retrotranscription of sst2 mRNA with external primer SST2/R (step a) and then amplified in the presence of both external primers (step b). Two separate PCR amplifications were performed, combining each external primer with the relative internal primer (steps c and d). The two tails of SST2/+1 and SST2/+2 internal primers are unrelated to the template but are complementary to each other, allowing the renaturation of the two resulting PCR products (step e) and the following PCR amplification with external primers (step f). The cDNA competitor was then cloned in a plasmid vector, and in vitro T7 run-off transcription of pSSR-I linearized with Sall generated the cRNA competitor (for more details see “Materials and Methods”).

Ten μl of each 100-μl reaction were run on a 12% polyacrylamide gel, stained with ethidium bromide, and quantified using a CCD video camera and image analysis as reported previously (32). The ratios between the two amplification products were then plotted against the amount of competitor molecules initially added to the sample, and the equivalence (i.e., the concentration of competitor giving a 1:1 competitor:target ratio) corresponded exactly to the number of mRNA molecules present in the initial sample (Fig. 2).

To confirm the specificity of the amplified product, Southern blot analysis was performed using a sst2-specific oligonucleotide probe derived from a region of sst2 mRNA internal to the sequence spanned by two external primers. The sequence of this probe was 5’-CGGCCAAGIGGAGGAGACCC-3’ (which corresponds to nucleotides 452–471 of sst2 mRNA).

DNA Competitor and Measurement of N-myc Amplification. N-myc oncogene copy number was determined in DNA extracted from cell lines and tumors using a standard phenol-chloroform procedure. The degree of N-myc amplification was determined with a two-step competitive PCR as described previously (32, 33) using a DNA multiple competitor (pONC) (34). In the first competitive PCR, we determined the exact concentration of extracted DNA by referring to the
amount of single-copy reference gene, \( \beta \)-globin, whereas in the second one the number of N-myc gene copies was evaluated. PCR was carried out in 50 \( \mu l \) of reaction mixture containing 2.5 units AmpliTaq DNA polymerase (Perkin Elmer-Cetus), 50 pmol of each primer, 200 nM each nucleotide (Boehringer Mannheim), and 1.5 mM MgCl\(_2\). Each PCR cycle for both \( \beta \)-globin and N-myc included 1 min at 95\( ^\circ \)C, 1 min at 60\( ^\circ \)C, and 1 min at 72\( ^\circ \)C. Following a 40-cycle PCR, an aliquot of each sample (10 \( \mu l \)) was electrophoresed on a 12% polyacrylamide gel, stained with ethidium bromide, and the intensity of the bands was analyzed using image analysis. In both cases the determination was performed by the addition of three dilutions of pONC multiple competitor, and the results were calculated on the basis of the densitometric ratios of competitor and genomic bands using the same procedure described for mRNA evaluation.

**Northern Blot Analysis of sst2 Expression in NB Cell Lines.** For each sample, 10–30 \( \mu g \) of total RNA were fractionated in a 1.2% agarose gel containing 8% formaldehyde. RNAs were then transferred onto nylon membranes (Hybond-N; Amersham, Milan, Italy) and baked at 80\( ^\circ \)C for 2 h. Membranes were prehybridized for 1 h and hybridized overnight at 65\( ^\circ \)C with the Church and Gilbert solution containing 10 mg/ml BSA, 7% SDS, 0.25 \( M \) sodium phosphate buffer, 1 \( M \) EDTA (pH 8), and 0.2 mg/ml hot, denatured sonicated herring sperm DNA. The probe for the detection of sst2 mRNA was derived from PCR amplification of CHP-404 cDNA with the same primers used for the competitive RT-PCR. The amplified DNA was precipitated and fractionated in a 2% agarose gel. The specific band was excised from the gel and purified from agarose using the Qiaex II agarose gel extraction kit (Qiagen, Heiden, Germany). To correct possible differences in RNA loading, the same nylon membranes were subsequently hybridized to rat cyclophilin cDNA (30).

The probes were labeled with deoxycytidine 5'-[\( ^{32} \)P]tri-

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**Fig. 3** Linear relationship between the log of SS-14-binding capacity and the log of the concentration of sst2 mRNA molecules as determined by competitive RT-PCR in six human NB cell lines. Values for SS-14-binding capacities were derived from radioligand-binding studies using SS-14 as tracer (16) with methods previously described (16). Insets, separate relationships for both low (upper inset) and high (lower inset) binding sites. The affinity constant, \( K_a \) (nm, mean \( \pm \) SE) of SS-14 for the high-affinity site was CHP-134B, 0.5 \( \pm \) 0.12 (\( n = 11 \)) and CHP-404, 0.76 \( \pm \) 0.48 (\( n = 6 \)) and was not measurable for the other cell lines. The affinity constant, \( K_a \) (nm, mean \( \pm \) SE) of SS-14 for the low-affinity sites was as follows: SK-N-AS, 116 \( \pm \) 41 (\( n = 6 \)); LA-N-5, 54 (\( n = 1 \)); SMS-KCN, 23 (\( n = 1 \)); CHP-134B, 78 \( \pm \) 21 (\( n = 11 \)); CHP-404, 68 \( \pm \) 47 (\( n = 6 \)); and SH-SYSY, 37 \( \pm \) 14 (\( n = 3 \)).

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Unpublished observation.
Northern blot analysis of sst2 expression and the concentration of both sites (55-14 high-affinity site) onto a nylon membrane, and then hybridized with a 32P-labeled probe for human sst2 gene (upper gel). Lower gel, reprobe of the Northern blot with a cyclophilin probe.

When 30 μg of total RNA derived from five NB cell lines were subjected to Northern blot analysis, we found the expected transcripts for sst2 (17) in only CHP-404 and CHP-134B (Fig. 4). Indeed, SH-SY5Y, SKN-AS, and SMS-KCNR were apparently negative. In addition, when a lower amount of CHP-404 RNA was used (10 μg), we obtained just a faint signal. Overall, these results are fully consistent with those from both RT-PCR and binding studies. Hence, by conventional Northern blot analysis we were able to detect a measurable signal only in those cell lines expressing the highest concentrations of receptors (higher than 7 × 10^7 molecules/μg RNA, as measured by RT-PCR). It is worthwhile noting that NB cells expressing a number of sst2 molecules/μg RNA higher than 7 × 10^7 were biologically responsive to SS-14 stimulation (16), expressing high-affinity SS-14-binding sites (16), and positive using Northern blot analysis, indicating that this number represents a sort of biological threshold for the activity of these receptors.

The mRNA levels of sst2 in 15 primary NB tumors were in the range of the levels observed in NB cell lines (9 × 10^4 to 4 × 10^7 molecules/μg RNA). Table 1 shows the characteristics of patients from whom the tumor specimens were taken as well as sst2 expression and degree of N-myc amplification. It is important to note that both the latter parameters have been quantitated for the first time with competitive RT-PCR. Although the number of patients in this study is very limited, it is intriguing that the lowest expression of sst2 was found in patients with N-myc amplification (a poor prognostic factor) and the highest expression was found in a patient with stage IVs disease (a spontaneously regressing form). In addition, we observed a statistically significant negative relationship between the degree of N-myc amplification and sst2 expression (P < 0.005). Conversely, the neuroendocrine marker SgII was positively related to sst2 expression (P < 0.05), whereas a lack of correlation was found between sst2 expression and chromogranins A and B. When patients were divided according to the presence of metastatic dissemination, we found that the lack of dissemination was associated with a higher expression of sst2 (6.2 × 10^6 molecules/μg RNA) than when metastases are present (3.33 × 10^7 molecules/μg RNA, P < 0.05). Furthermore, tumors with more benign histopathological features show a higher expression of sst2 than tumors with poor pathological grading, according to the Shimada et al. (19) classification (differentiating, 4.13 × 10^6 molecules/μg RNA; undifferentiated, 4.08 × 10^7 molecules/μg RNA, P < 0.05).

Fig. 5A shows the estimated progression-free survival for patients with NB as a function of sst2 expression in the primary tumor. Since we previously demonstrated that the cutoff of 7 × 10^6 molecules/μg RNA divided SS-14-responsive from SS-14-unresponsive NB cell lines, we used this threshold of sst2 gene expression for evaluation of progression-free survival. Progression-free survival was 87.5% if sst2 expression was above 7 × 10^6 molecules/μg RNA and 12.5% if sst2 expression was below this threshold (P = 0.01). For comparison, Fig. 5B displays the Kaplan-Meier survival curves for the same patients according to N-myc amplification. As previously reported, N-myc amplification is a poor prognostic factor for NBs (37, 38). Indeed, patients with N-myc amplification show a dramatically worse progression-free survival rate than patients with a single copy of this oncogene (P < 0.00005).

**DISCUSSION**

In this study, we describe for the first time the quantitation of sst2 gene expression as applied to NB cell lines and primary tumors and its relevance for prognostic purposes. To detect and quantitate sst2, we developed a competitive RT-PCR method that entails coamplification of sst2 and a dilution series of an RNA competitor. The RNA competitor has been designed as an insertion mutant of the sst2 mRNA target, easily distinguishable with PAGE and ethidium bromide staining from the natural transcript counterpart because of the addition of a short unre-
lateral nucleotide sequence. At the end of the amplification reaction, we estimated the relative concentrations of target cDNA by the evaluation of the ratios between the two PCR products (competitor and target cDNAs). The use of an RNA competitor, as an internal standard, enables us to compensate for the variability of the RT and the subsequent PCR, as well as allowing accuracy of the assay after extended cycling. Therefore, competitive RT-PCR provides accurate determination of the absolute amount of specific sst2 mRNA over a wide range of concentrations, spanning at least six log units.

According to a previous semiquantitative report in the rat (1), we found that in humans the lowest abundance of sst2 gene expression is in the testis (3 × 10^6 molecules/μg RNA), whereas the highest levels are found in the adrenal gland (6 × 10^7 molecules/μg RNA). In NB cell lines, the relative abundance of sst2 mirrors the binding capacity of SS-14. In CHP-404 and CHP-134B cells, the expression of sst2 is in the same order of magnitude as the level found in the adrenal gland. Interestingly, we previously reported that these cell lines express an elevated concentration of high- and low-affinity sites for SS-14 and octreotide and are responsive in vitro to the stimulation by these agonists (16). Indeed, in these cell lines, both SS-14 and octreotide elicited a decrease in either forskolin- or vasoactive intestinal peptide-stimulated cAMP levels; a dose-dependent perturbation in intracellular calcium mobilization, and a decrease in DNA synthesis through an accumulation of cells in G_1 (16). Subsequent experiments with sst2-transfected cells clarified that almost all of these effects are indeed mediated by sst2 (1, 2, 11, 40). In the present study, we also found specific transcripts for sst2 (even if with a lower degree of expression than in the adrenal gland, i.e., one or two log units) in other NB cell lines, which we previously reported as being devoid of high-affinity binding sites, unresponsive to SS receptor stimulation (16). These transcripts were also undetectable using Northern blot analysis (present study). It is interesting to note that in all of these cell lines, we detected the presence of low-affinity SS14-binding sites (Ref. 16; present study). We have now proved that in SMS-KCNR, SK-N-AS, LA-N-5, and SH-SY5Y, the capacity of the low-affinity sites is positively correlated with the expression of sst2, as determined by competitive RT-PCR. This finding suggests that even the low-affinity site possibly corresponds to sst2. However, the interpretation of this result is rather difficult. First, we need to consider that NB cells might express multiple subtypes of SS receptors not investigated in the present study. However, all of the SS isoreceptors described display high affinity for SS-14 but variable affinity for octreotide. Another possibility is that the low-affinity sites identified by binding studies represent sst2 receptors (or other subtypes) that were found in the low-affinity state because of G-protein coupling. We used only SS receptor agonists which are very sensitive to GIP-binding interferences as radioligands (specific antagonists are not available, as far as we know) The relatively lower sensitivity of radioligand-binding studies versus RT-PCR for the detection of the high-affinity sites could be another explanation. In other words, since the low-affinity sites are many times more concentrated than the high-affinity ones, they are more easily measurable by radioligand-binding studies. In addition, a low-affinity binding site concentration is more readily compared with results from RI-PCR for the detection of the high-affinity site possibly corresponds to sst2. However, the interpretation of this result is rather difficult. First, we need to consider that NB cells might express multiple subtypes of SS receptors not investigated in the present study. However, all of the SS isoreceptors described display high affinity for SS-14 but variable affinity for octreotide. Another possibility is that the low-affinity sites identified by binding studies represent sst2 receptors (or other subtypes) that were found in the low-affinity state because of G-protein coupling. We used only SS receptor agonists which are very sensitive to GIP-binding interferences as radioligands (specific antagonists are not available, as far as we know) The relatively lower sensitivity of radioligand-binding studies versus RT-PCR for the detection of the high-affinity sites could be another explanation. In other words, since the low-affinity sites are many times more concentrated than the high-affinity ones, they are more easily measurable by radioligand-binding studies. In addition, a low-affinity binding site concentration is more readily compared with results from RT-PCR, a very sensitive assay. Nevertheless, our results indicate that above the threshold of 10^8 molecules/μg RNA of sst2, radiolabeled octreotide and SS-14 detect the presence of high-affinity binding sites coupled to a physiological responsiveness. Below this threshold there is a shadowed area that needs to be clarified by combined competitive RT-PCR, binding, and functional studies in sst2-transfected cells with different gene expression.

The most important finding of the present study is the

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<th>CgB</th>
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<th>Site</th>
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<td>61</td>
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<td>-</td>
<td>Diff.</td>
<td>II</td>
<td>NED 71</td>
</tr>
<tr>
<td>14</td>
<td>1 × 10^6</td>
<td>1</td>
<td>113</td>
<td>42</td>
<td>72</td>
<td>F/29</td>
<td>Pelvis</td>
<td>-</td>
<td>Diff.</td>
<td>III</td>
<td>NED 90</td>
</tr>
<tr>
<td>15</td>
<td>4 × 10^6</td>
<td>1</td>
<td>237</td>
<td>11</td>
<td>103</td>
<td>M/I</td>
<td>Adrenal</td>
<td>+</td>
<td>Diff.</td>
<td>IVs</td>
<td>NED 91</td>
</tr>
</tbody>
</table>

* Absolute values are expressed as arbitrary units of absorbance by measuring Northern blot signal intensities with laser densitometer.
  † MTS, metastases at diagnosis; Diff., differentiating histology; Undiff., undifferentiated histology; DOD, dead of disease; NED, no evident disease; AWD, alive with disease; M, male; F, female; n.d., not done.
  ‡ According to Shimada et al. (19).
A perspective role of sst2 gene expression as a new prognostic factor for NB tumor. Although our investigation is limited to a relatively low number of observations, we found that tumors with a high degree of sst2 expression show a more favorable survival rate than tumors with a lower expression. Moreover, the expression of sst2 is significantly lower in undifferentiated and metastasizing tumors than in tumors with more benign clinical and pathological characteristics. Accordingly, the amount of sst2 expression is negatively related to the degree of the amplification of the oncogene N-myc, also assessed by competitive PCR. N-myc is a member of the myc gene family in which the degree of amplification greatly contributes to the malignant phenotype of NB cells (38) and has a definitive poor prognostic role with regard to patient outcome (Ref. 39; see also present results). It is worth mentioning that, in our small sample size, tumors with levels of sst2 gene expression below $10^7$ molecules/µg RNA show N-myc amplification. Conversely, the highest level of sst2 (4 × $10^9$ molecules/µg RNA) was found in one primary tumor in a patient who later showed metastatic dissemination, but had been classified as stage IVs according to the Evans et al. classification (20). In patients at stage IVs, cure occurs without any or limited therapy due to spontaneous regression of all of the lesions as part of the natural history of this disease (41). Furthermore, we also found a positive correlation between sst2 gene expression and SglII gene expression, as detected using Northern blot analysis (22). Indeed, SglII is a natural constituent of chromaffin granules that is up-regulated in NB cells undergoing neuroendocrine differentiation (42). In addition, a previous report indicated that a higher relative expression of this neuroendocrine marker is associated with a better prognosis in NB tumors (22). Hence, our results, although preliminary, suggest that sst2 gene expression may identify a subset of NB tumors with a more differentiated phenotype and, thus, could represent a new prognostic factor for this disease. Since our study population is relatively small, it is not possible to establish whether or not sst2 gene expression represents a new N-myc oncogene-independent prognostic indicator for patient outcome. Hence, our results need to be confirmed in larger population studies before they can be of clinical value. Our results agree with previous reports based on the qualitative (43) or quantitative (44) detection of SS-binding sites in NB tumors. Indeed, both studies showed that the presence of binding sites for radiolabeled SS-14 and analogues represents a favorable prognostic factor for NB tumors, since it was positively related to survival. However, in both studies a consistent part of the samples investigated were found to be SS receptor negative. Conversely, we found expression of at least one subtype of SS receptors, sst2, in all of the tumors investigated, although with different degrees of expression. As previously mentioned, discrepancy may be related to the different target (protein versus mRNA) and sensitivity of the assay. In conclusion, our study extends previous observations on the presence of SS receptors in NB cells and tumors (16, 43, 44) to the quantitative evaluation of sst2 gene expression using the rather sensitive and accurate technique of competitive RT-PCR. Therefore, our study fulfills some of the recommendations for further investigation expressed by the Tumor Receptor Imaging Workshop (45). At this meeting, studies on the correlation between SS receptor protein and its relative gene expression in tumor samples were strongly recommended. Our results indicate that the prognostic value of sst2 levels in NB tumors is worth evaluating in a larger series. In addition, since sst2 binds octreotide with high affinity, it is possible that the $^{111}$In-labeled octreotide analogue (Octreoscan) would be useful not only for the in vivo visualization of NBs but also as a prognostic tool. Therefore, SS receptor visualization with Octreoscan may be a cost-effective alternative to other imaging in NB.

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