Measurement of somatostatin receptor subtype 2 mRNA in breast cancer and corresponding normal tissue

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Abstract

Somatostatin analogs are effective in inhibiting growth of human breast cancer cell lines. These antiproliferative effects are mediated by specific receptors located on cell membranes. The somatostatin receptor subtype 2 (sst2) is the principal mediator of somatostatin effects in normal and cancer cells, and its presence has already been demonstrated in breast cancer. The purpose of our study was to evaluate the clinical relevance of the expression of sst2 by quantifying its mRNA in a large group of infiltrating breast cancers and their corresponding normal tissues.

The expression of sst2 mRNA was measured with quantitative real time RT-PCR in 169 breast cancers and in their corresponding unaffected tissues. We evaluated the association of sst2 expression with the commonest clinical-pathologic features of breast cancer. The correlation with a marker of cell proliferation (Ki-67) and with receptor concentration was also evaluated.

In cancer tissues, we found that the absolute concentrations of sst2 mRNA were significantly higher in estrogen receptor (ER)-positive samples \((P = 0.002)\) as well as in lymph-node-negative cancers \((P = 0.04)\) (Student’s t-test or one-way ANOVA). In addition, sst2 mRNA was significantly higher in breast cancers than in corresponding unaffected tissues \((P = 0.0002)\). However, when the clinical-pathologic parameters were considered, this gradient maintained its statistical significance only in tumors expressing positive prognostic markers, such as the presence of ER \((P = 0.0005)\) and progesterone receptors (PgR) \((P = 0.005)\), and the lack of lymph-node involvement \((P = 0.0003)\). The same difference was also significant in postmenopausal women \((P = 0.001)\) and in T1 patients \((P = 0.001)\). In addition, sst2 mRNA expression was significantly higher \((P = 0.008)\) in low-proliferating breast cancers. Finally, we found that the quantitative expression of sst2 mRNA was directly related to the PgR concentration in breast cancer tissues \((P < 0.001)\).

Our data seem to indicate that an upregulation of sst2 gene expression is a common feature of breast cancers which, on the basis of conventional predictive parameters, are expected to have a better prognosis. Featuring a possible role of somatostatin analogs in combined endocrine therapies for breast cancer, our results seem to confirm that the sst2 status of the tumor should be previously investigated.

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Introduction

Breast cancer is the most frequently diagnosed cancer among women in the West. In Italy, the number of new cases every year is approximately 30,000, with an estimated lifetime risk of developing such neoplasia of 1 in 14 women (Sant et al. 2001). Since mortality is
about one-third of the incidence, breast cancer currently represents a leading cause of cancer death in women. The ultimate goal in studying breast cancer biology is to reduce mortality by predicting the prognosis of existing disease, but first of all by improving the response to different therapies (Nass et al. 1999). Many morpho-pathologic and biologic parameters have been identified as important factors for patient prognosis and for selection of therapy, such as tumor size, grading, axillary lymph-node involvement, and estrogen receptor (ER) and progesterone receptor (PgR) status (Harris et al. 1997). Despite this, it is important to identify new prognostic factors, in order to select patients at different risk of recurrence, and to improve traditional treatment strategies and increase overall survival.

The characterization of somatostatin (SS) receptor expression may be able to give further insights into conventional prognostic and therapeutic approaches to breast cancer. SS is a widely distributed, multifunctional inhibitory peptide hormone which is involved in multiple cellular activities. In particular, SS regulates cell secretion and proliferation through a family of specific cell-surface receptors (ssts) (Patel & Srikant 1997). Growth inhibition by SS and its analogs can occur both indirectly, through the modulation of trophic peptide hormones and growth factors, as well as through inhibition of angiogenesis (Woltering et al. 1990), and directly, through interaction with SS receptors located on tumor cell membranes (Patel & Srikant 1997). Five distinct SS receptor subtypes (sst1–sst5) have been identified, cloned and sequenced (Yamada et al. 1992a,b, 1993). Among these, sst2 mediates the antiproliferative effect more efficiently than the others (Buscail et al. 1995) and shows the highest affinity for the SS analog, octreotide (Patel & Srikant 1997). Cellular ssts are present in many endocrine and neuroendocrine tumors (benign and malignant) and provide a basis for their diagnosis and treatment (Robbins 1996). In addition, the presence of ssts has also been reported in a large number of human primary non neuroendocrine tumors, such as breast cancer (Evans et al. 1997, Schaer et al. 1997). Indeed, expression of ssts in human primary breast cancer has a very high incidence, and it has been demonstrated by biochemical cross-linking techniques (Prevost et al. 1993), in vitro autoradiography (Papotti et al. 1989, Reubi & Torhorst 1989, Reubi et al. 1990, van Eijck et al. 1994) and in vivo scintigraphy (Krenning et al. 1993, van Eijck et al. 1994), using various SS analogs. Some studies have also been performed to characterize the pattern of expression of different ssts subtypes in breast carcinoma (Reubi et al. 1994, 2001, Evans et al. 1997, Schaer et al. 1997, Schulz et al. 1998). The majority of them showed that sst2 was the most frequently expressed ssts subtype in the tumor samples. Finally, studies on cell lines have indicated that SS analogs are effective in inhibiting growth of various human breast cancer cells, including MCF-7 and T45D both in vivo and in vitro (Setyono-Han B et al. 1987, Scambia et al. 1988, Nelson et al. 1989, Pagliacci et al. 1991, Prevost et al. 1991, Weckbecker et al. 1992).

All this experimental evidence has prompted few clinical trials to evaluate the efficacy of SS analogs in the treatment of human breast carcinoma. It has to be remarked that up to now SS analog therapy has been disappointing in the management of advanced malignancies, and improvements are likely to come only from such analogs conjugated with cytotoxic agents or radio-emitting molecules (McCarthy et al. 1998, Nagy et al. 1998, Shally & Nagy 1999, Jenkins et al. 2001). Some of the studies showed a reduction in terms of IGF-I plasma concentrations, but no effects on tumor progression (Manni et al. 1989, Vennin et al. 1989, O’Byrne et al. 1999). Two recent trials (Ingle et al. 1999, Bajetta et al. 2002) showed that the combination of tamoxifen plus octreotide was substantially as efficacious as tamoxifen alone in the treatment of advanced breast carcinoma. Indeed, in none of the aforementioned studies has the ssts pattern of tumors been investigated, knowledge of which is critical if the therapeutic effects of SS analogs are to be exploited.

In addition, ssts scintigraphy has been shown to be effective in the detection of primary breast cancer, as well as of ssts-positive breast cancer recurrence (van Eijck et al. 1994). This technique may also be of value in selecting patients for clinical trials with SS analogs. Finally, our previous experience on neuroblastoma (Casini Raggi et al. 2000) clearly showed that the quantitative determination of sst2 gene expression might be able to give relevant insights in terms of overall and disease-free survival. Interestingly, Foekens et al. (1990) already observed a significantly longer relapse-free survival for patients with ssts-positive breast cancer than for patients with ssts-negative tumors, suggesting that sst2 expression may represent a prognostic factor also for this tumor.

In an attempt to elucidate the reasons of the disagreement between the aforementioned in vitro studies and the clinical results obtained up to now, we measured sst2 mRNA expression in a large number of surgically removed breast carcinomas with an accurate quantitative RT-PCR method based on TaqMan reaction (Pinzani et al. 2001). Moreover, sst2 mRNA expression was quantified in paired normal tissues to evaluate the different expression of sst2 between tumoral and normal breast tissue. The association between sst2 mRNA expression and the clinical-pathologic parameters commonly used for breast cancer was then assessed.
In an attempt to clarify the relationship between sst2 mRNA expression and cell proliferation, the expression of Ki-67, a well-known marker of cell proliferation, was evaluated by immunohistochemistry in the same group of patients, and then correlated with the expression of sst2 mRNA.

Finally, we investigated the relationship between sst2 gene expression and ER status, as the association between these two biologic features has been clearly demonstrated in vitro (Xu et al. 1996, 1998).

Materials and methods

Patients

Tissues were obtained from 169 patients with invasive breast cancer undergoing surgery in the Surgical Department of Careggi Hospital (Florence, Italy). The age of patients ranged from 30 to 88 years (mean age: 58); among them, 51 were in premenopausal status. On the basis of TNM staging, 117 tumors were classified for tumor size (T) as T1, 44 as T2, one as T3 and seven as T4. Seventy-two patients showed axillary lymph-node involvement (N+), whereas 97 patients were classified as N0. According to the histopathologic Nottingham grading (Elston & Ellis 1991), for their grading index (G) 48 tumors were classified as G1, 53 as G2 and 68 as G3. The evaluation of steroid receptors showed 136 ER-positive tumors, while 107 were positive for progesterone receptors. From each patient, a random sample was collected from the center of the tumor after dividing it at the maximum diameter. An unaffected tissue sample was chosen either from the normal tissue adjacent to the quadrant bearing the tumor \( n = 128 \), or from the opposite quadrant in case of mastectomy \( n = 41 \). No difference was observed in these two groups in terms of sst2 expression. Samples were immediately snap-frozen and stored in liquid nitrogen before RNA extraction. Total RNA was extracted from each sample with RNeasy Kit (Quiagen, Milan, Italy). Since sst2 is an intronless gene, each RNA sample was first submitted to a conventional PCR with the same primers and cycling conditions to be used in the PCR reaction to generate the reference mRNA. An amount of 1 μg of total RNA was reverse-transcribed by random exomers, following a classical reverse-transcription 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 of \( C_t \) values generated from initially known concentrations of RNA extracted from neuroblastoma cell line CHP404, which overexpresses sst2 mRNA. An amount of 1 ng of total RNA was reverse-transcribed, and cDNA was then serially 1:10 diluted to obtain five standard concentrations to be used in the PCR reaction to generate the reference curve (Pinzani et al. 2001). All determinations were performed in triplicate.

Immunohistochemistry for ER, PgR and Ki-67

Formalin-fixed, paraffin-embedded specimens of the corresponding 169 primary breast tumors were used for immunohistochemical analysis. Serial sections, 5 μm thick, were mounted on electrostatic slides, air dried overnight at 37 °C, deparaffinized in xylene and rehydrated in graded alcohol. Endogenous peroxidase activity was blocked by immersion in a solution of 3% H₂O₂ in methanol for 20 min.

Antigens were retrieved by placing the sections in boiling citrate saline buffer (10 mM, pH 6.0) for 40 min in a microwave oven at 300 W. Sections were left to cool in the buffer at room temperature for 20 min.

After washing in PBS solution (pH 7.4), the slides were covered with normal serum (Lab Vision Corporation, Fremont, CA, USA) to block nonspecific binding sites.

The following primary antibodies were applied and incubated overnight at 4 °C: anti-ER, clone 1D5 (BioGenex, San Ramon, CA, USA), diluted 1:30; anti-PgR, clone 1A6 (BioGenex), diluted 1:50; and anti-Ki-67, clone Mib-1 (Immunotech, Marseille, France), diluted 1:60.
After washing in PBS, the primary antibody was detected by incubation with biotinylated antimouse immunoglobulin (Ig) G (Lab Vision) for 15 min at room temperature and by incubation with the streptavidin–biotin–peroxidase complex reagent (Lab Vision) for 15 min at room temperature. The immunostaining was visualized with diaminobenzidine-hydrogen peroxidase (BioGenex). Sections were counterstained lightly with Mayer’s hematoxylin. After dehydration in graded alcohol and xylene, slides were mounted with Permount (Fisher Scientific, Fair Lawn, NJ, USA). Negative control slides were obtained by omitting the primary antibody.

Sections of breast carcinomas with known positive expression were used as positive control for ER, PgR and Ki-67 staining. Immunocytochemical results were evaluated in double blind by two pathologists (S.B. and V.V.). ER, PgR, and Ki-67 expression was scored as the percentages of positive nuclei over the total number of cancer cell nuclei counted. Discordant cases (less than 10% for each parameter) were discussed, and a consensus statement was reached.

For ER and PgR status, only two categories were considered (positive/negative), according to well-established cutoff values (10% for both ER and PgR) (Balaton 1999). Two categories were used (low/high) for Ki-67, according to a well-established cutoff value (20%) (Bouzubar et al. 1989, Veronese et al. 1993).

### Binding analysis of ER and PgR

Total proteins were also extracted in a subset of patients (n = 73), when permitted by the dimension of breast samples. PgR content was then assayed in breast cancer and in the corresponding unaffected tissues with a radioligand assay, with the method suggested by the European Organization of Research and Treatment of Cancer (EORTC, 1980).

### In situ hybridization for sst2 expression

**In situ** hybridization was performed according to a technique described elsewhere (Romagnani et al. 2001). Briefly, 5 μm frozen sections were cut and fixed in 4% paraformaldehyde for 20 min. After a prehybridization treatment (0.2 M HCl, 0.125 mg/ml pronase, 4% paraformaldehyde, acetic anhydride 1:400 in 0.1 mol/l triethanolamine buffer, pH 8), sections were dehydrated in increasing ethanol concentrations. A volume of 30 μl hybridization solution (4 standard saline citrate, 1 Denhardt’s solution, 10% dextran sulfate, 0.1 mg/ml sheared herring sperm DNA and 1 mg/ml yeast tRNA), containing 8 x 10⁵ counts/min 35S-labeled human sst-2 RNA probe, was applied on each section and covered with Parafilm. Human sst-2 RNA probe was synthesized as previously described (Romagnani et al. 2001). cDNA was subcloned in PGEM-4Z plasmid vector, by incubating with T4 ligase (Gibco-BRL, Life Technologies, Berlin, Germany) at 15°C for 4 h. The cDNA was subsequently linearized with HindIII or XbaI restriction enzymes followed by phenol–chloroform extraction and ethanol precipitation. Thereafter, sense and anti-sense RNA-radiolabeled probes were synthesized using SP6 or T7 RNA polymerases as appropriate (Riboprobe Gemini System, Promega Italy, Milan, Italy), in the presence of 35S-labeled thioUTP (1300 mCi/mmol; NEN-Du Pont, Paris, France). RNA probes were extracted with phenol–chloroform, ethanol precipitated, and subsequently subjected to alkaline digestion. Hybridization was performed at 52°C for 16 h. After that, sections were washed and autoradiography was performed. Exposure time varied from 15 to 20 days. Sections were developed in D19, fixed in Kodak fixative, counterstained with hematoxylin–eosin–phloxine and mounted with Permount. Negative controls consisted of sections hybridized to a sense RNA probe.

### Statistical analysis

Statistical analyses were performed with software from SPSS (Chicago, IL, USA). Significance of the differences was evaluated by Student’s t-test for paired or independent samples, or by one-way ANOVA, as indicated.

### Results

#### Relationship between sst2 mRNA expression in breast cancer and clinical-pathologic features

All breast cancers analyzed with real-time RT-PCR expressed sst2 mRNA. In Table 1, we reported the mean values of sst2 mRNA expression in tumor tissues, in relation to the commonest clinical-pathologic features of breast cancer. We found that sst2 mRNA was significantly higher in node-negative patients (mean ± SE: 8.7 ± 2.0 x 10⁶ molecules/μg total RNA) than in patients with lymph-node involvement (3.6 ± 0.9 x 10⁶ molecules/μg total RNA, P = 0.043). Similarly, sst2 expression was significantly higher in ER-positive (7.6 ± 1.6 x 10⁹ molecules/μg total RNA) than in ER-negative cancers (2.4 ± 0.5 x 10⁹ molecules/μg total RNA; P = 0.002). In addition, PgR-positive cancers (8.3 ± 1.8 x 10⁹ molecules/μg total RNA) showed a trend to a higher expression of sst2 mRNA when compared to PgR-negative tumors (3.9 ± 1.6 x 10⁹ molecules/μg total RNA), although this difference was not statistically significant (P = 0.069). We did not find any relationship between sst2 mRNA
expression and the other clinical-pathologic features (menopausal status, tumor size, grading and histotype).

Comparison of sst2 mRNA expression in breast cancer and corresponding unaffected tissues

The expression of sst2 mRNA in the corresponding normal breast tissues also showed considerable variability. Table 1 shows the values obtained in both affected and unaffected samples. Overall, sst2 mRNA was more expressed in tumoral tissues $(6.5 \pm 1.2 \times 10^6)$ than in the corresponding unaffected tissues $(1.1 \pm 0.4 \times 10^6$, $P = 0.0002)$. Stratifying patients according to the most important clinical-pathologic parameters, we found that in ER-positive as well as in PgR-positive cases the expression of sst2 mRNA was significantly higher in tumoral samples than in the corresponding normal tissues ($P = 0.0005$ for both comparisons), whereas the same difference was not significant in either the ER-negative or the PgR-negative group. Similarly, the difference in sst2 mRNA expression between tumors and the corresponding normal tissues was significant in node-negative patients ($P = 0.0003$), but not in patients with lymph-node involvement. In addition, this difference was significant in T1 tumors ($P = 0.001$), but not in the mixed T2+ group. The same difference was also highly significant in the G1 tumors ($P = 0.009$), as well as in the G2–G3 group ($P = 0.008$). We also observed that the difference in sst2 mRNA expression in paired samples was significant in postmenopausal ($P = 0.001$) but not in premenopausal women. Finally, according to the histotype, the difference

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<th>Table 1 sst2 mRNA expression in paired breast cancers and corresponding normal tissues and clinical features of patients.</th>
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<td>Ductal plus lobular</td>
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<td><strong>ER status</strong></td>
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*Molecules of sst2 mRNA/μg total RNA ($\times 10^6$).

*Paired t-test (cancer vs corresponding noncancer tissue).

*t-test for unpaired samples.

ANOVA test.
was statistically significant in the group of invasive ductal breast carcinomas ($P = 0.001$), but not in the other histologic types.

**sst2 mRNA expression and proliferation in breast cancer**

Since the antiproliferative action of SS in human tumors is well known, in the same group of patients we evaluated the relationship between sst2 mRNA expression and the expression of Ki-67, a well-known marker of cell proliferation. According to our results, sst2 gene expression ($9.5 \pm 2.0 \times 10^6$) was significantly higher ($P = 0.008$) in tumors with a low proliferation index, assessed by Ki-67 expression ($n = 109$; Ki-67 positivity $\leq 20\%$), than in tumors with a high proliferation index ($n = 60$; Ki-67 $> 20\%$) ($2.1 \pm 0.3 \times 10^6$), as shown in Fig. 1.

**sst2 mRNA expression and steroid receptors**

Using serial sections of breast tumors, we observed that ER and sst2 mRNA were clearly localized in the cells of glandular epithelium (Fig. 2). The sections are derived from a tumor expressing PgRs, ERs, no lymph-node involvement and high levels of sst2 mRNA. This finding might suggest colocalization of the two receptors and their mutual interaction.

Since the expression of progesterone receptors is a well-known biologic marker of estrogen activity, we determined the concentration of progesterone receptors in a subset of breast cancer tissues, using a quantitative radioligand assay (Veronese et al. 1993). We found that the expression of sst2 mRNA in tumoral tissues was directly related ($P = 0.003$) to the PgR density (Fig. 3).

**Discussion**

The clinical and pathologic relevance of SS receptors in breast cancer patients is still unclear. In a small group of breast cancer patients, Foekens et al. (1990) found a direct relationship between presence of SS receptors and 5-year disease-free survival. More recently, Pilichowska et al. (2001) found that sst2 immunoreactivity was significantly associated with a lower histologic grade in breast cancer. As previously mentioned, the antiproliferative action of SS analogs on breast cancer cell lines has been clearly demonstrated, but the clinical studies performed up to now have given controversial results and do not support the routine use of such analogs in the treatment of nonendocrine tumors such as breast cancer.

Taking into account the heterogeneity between and within individual tumors with respect to density of ssts-binding sites, the aim of our study was to provide a quantitative estimation of sst2 mRNA expression in a large group of invasive primary breast cancers. We have already demonstrated, in other human cancer models, that the measurement of sst2 mRNA expression with quantitative PCR appears to be well correlated to the *in vitro* and *in vivo* expression of the related protein (Sestini et al. 1996, Briganti et al. 1997, Casini Raggi et al. 2000, 2002). In addition, as recently demonstrated in colon cancer (Casini Raggi et al. 2002), we compared the results obtained in tumoral tissues with those obtained in the corresponding unaffected tissues. This approach, beside providing the absolute value of sst2 gene expression in the tumor, also allows us to estimate its possible variations in comparison to the corresponding normal tissue, which represents the individual control sample.

Our data show that sst2 mRNA is expressed in all the tumor samples analyzed, as well as in their normal counterparts, although this finding was not confirmed by
Figure 2 Representative example of expression of sst2 mRNA and estrogen receptor (ER) expression in a tissue specimen obtained from one patient with progesterone- and estrogen-positive breast cancer, without lymph-node involvement and high levels of sst2 mRNA ($1.19 \times 10^5$ molecules/µg total RNA). (A) High signal in specimen from a patient with breast cancer hybridized with antisense sst-2 mRNA probe ($\times 400$). (B) Absence of signal in a section hybridized with a sense sst-2 mRNA probe ($\times 400$). (C) High immunoreactivity for ER in a tissue specimen obtained from a patient affected by breast cancer (red color) ($\times 400$). (D) Absence of ER signal in the same tissue specimen immunostained with an isotype-matched control antibody with irrelevant specificity ($\times 400$). Both positive signals are clearly localized in the glandular epithelium.

Figure 3 Linear relationship of sst2 mRNA expression with PgR concentration in 73 breast cancers.

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any protein assessment. On average, the expression of sst2 mRNA appeared to be significantly higher in tumoral than in normal tissues, but we observed a large interindividual variation of the sst2 mRNA ratio between the two samples. In particular, it has to be remarked that the aforementioned statistically significant difference was maintained only in tumors expressing better prognostic features, as observed in stratifying patients according to the most important clinical-pathologic parameters.

Since sst2 is considered the principal mediator of the antiproliferative effects of SS, an inverse relationship between sst2 expression and cancer cell proliferation would be expected. Indeed, our results show that tumors with a low proliferation index, as assessed by the expression of Ki-67, express significantly higher amounts of sst2 mRNA than tumors with a high proliferation index. This is, to our knowledge, the first direct in vivo demonstration of a direct relationship between sst2 expression and cancer proliferation.

The in vivo relationship between the ER status and sst2 gene expression was another aspect that we investigated. As already mentioned, the relationship between estrogens and sst2 has been already demonstrated in vitro experiments by Xu et al. (1996, 1998, Kimura et al. 2001), who also identified an estrogen responsive element in the sst2 promoter (1998). Our data indicate a clear in vivo linear relationship between a marker of estrogen activity (represented by the PgR density) and sst2 mRNA expression. Indeed, the PgR gene also presents an estrogen-dependent, positive regulating sequence in its promoter (Savouret et al. 1991). An additional confirmation of this relationship may also be obtained by the demonstration of the colocalization of sst2 mRNA and ERs in the cells of glandular epithelium.

In conclusion, our data seem to indicate that breast cancers that, on the basis of conventional predictive parameters, are expected to have a better prognosis, show an upregulation of sst2 mRNA expression. This linkage is particularly evident in relation to the lymph-node status and the steroid receptor expression, as well as according to the proliferative activity.

These findings cannot answer the question of whether sst2 expression may represent a new marker of breast cancer differentiation (sst2 as a tumor marker), or whether we can hypothesize an active role of sst2 in inhibiting breast cancer progression (sst2 as an oncosuppressor gene). In support of this latter hypothesis, a recent study in allograft models (Benali et al. 2000) demonstrated that stable expression of sst2 can inhibit both tumor growth and metastatic progression. In addition, the same group of investigators (Torrisani et al. 2001) described a functionally active polymorphism in the sst2 gene promoter, which can inhibit gene transcription in human pancreas adenocarcinomas. This and/or other epigenetic mechanisms, such as methylation of the sst2 promoter, may be responsible for a reduced expression of this receptor.

Finally, in suggesting a possible role of SS analogs in combined endocrine therapies for breast cancer, our results seem to confirm that the sst2 status of tumors should be elucidated before initiation of therapy in patients enrolled in clinical trials, and that therapies based on ER antagonists may influence the potentially therapeutic effects of SS analogs in this kind of neoplasia.

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