The antiproliferative effects of somatostatin receptor subtype 2 in breast cancer cells

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Aim: Somatostatin receptor subtype 2 (SSTR2) is the principal mediator of somatostatin’s (SST) antiproliferative effects on normal and cancer cells. Therefore, we investigated whether the enhanced expression of SSTR2 could inhibit the proliferation of tumor cells, and, if so, the mechanisms that might be involved.

Methods: SSTR2 expression levels were determined by qRT-PCR in several tumor cell lines. Then, a plasmid pIRES2-EGFP-SSTR2 (pSIG) was constructed and stably transfected into MCF-7 cells (MCF-7/pSIG). After SSTR2 overexpression was identified by qRT-PCR, immunofluorescence staining and a receptor binding assay, the MCF-7/pSIG cells were analyzed by PI staining for apoptosis and cell cycle arrest was tested by flow cytometry for epidermal growth factor receptor (EGFR) expression. The EGF-stimulated proliferation of MCF-7 cells was assayed by MTT.

Results: The human breast cancer cell line MCF-7 expresses a lower level of SSTR2, thereby partly accounting for the decreased response to SST. The overexpression of SSTR2 in MCF-7 cells resulted in apoptosis, cytostasis and G1/S cell cycle arrest. Furthermore, the expression of EGFR, together with EGF-stimulated proliferation, was markedly decreased in the MCF-7/pSIG cells.

Conclusion: Enhanced SSTR2 expression played an antiproliferative role in MCF-7 cells through inducing apoptosis and G1/S cell cycle arrest, and also by decreasing EGFR expression, thereby counteracting the growth-stimulating effect of EGF. Our data seem to indicate that developing a new therapeutic agent capable of upregulating SSTR expression could potentially be a way to block tumor progression.

Keywords: somatostatin; somatostatin receptor; epidermal growth factor receptor; breast cancer

Introduction
Somatostatin (SST) is an endogenously produced peptide elicits both direct and indirect effects on tumor biology. The indirect biological effects of SST or its analogs involve the inhibition of hormones and growth factors that promote tumor growth, while the direct effects involve the inhibition of proliferation and/or the induction of apoptosis via interactions with a family of G protein-coupled receptors (GPCRs) with five known subtypes (SSTR1-5) present in tumor cells. These five distinct SSTRs are distributed in human organs and tumors in a subtype-selective and tissue-specific fashion. Among them, SSTR2 shows the highest affinity for SST analogs and elicits the strongest antiproliferative effect. The SSTR2 was also proven to be the subtype mainly responsible for the high ¹¹¹In-DTPA-octreotide uptake in primary breast cancer.

Several recent reports demonstrated that SSTR expression in breast cancer is down-regulated either in more aggressive, less differentiated tumors or upon antiestrogen treatment, which could account for the ineffectiveness of SST treatment of breast tumors in clinical trials. In an attempt to explore whether breast cancer could respond to SSTs after improvement of the SSTRs, plasmid pSIG bearing the SSTR2 gene was constructed to transfect the MCF-7 cell line. Then, the antiproliferative effect of SSTR2 on these cells was investigated.

It is believed that less aggressive and well-differentiated tumors express SSTRs and have better long-term survival rates, whereas many undifferentiated tumors express ErbBs and have poor prognoses, indicating an inverse relationship...
between SSTRs and ErbBs. Moreover, SSTRs have been established to be colocalized with ErbBs in MCF-7 and MB-MDA 231 breast cancer cells\(^\text{80}\). On the basis of this evidence, we attempt to clarify the interaction between SSTR2 and EGFR, particularly regarding the expression of EGFR and the EGF-stimulated proliferation of MCF-7/pSIG cells.

**Materials and methods**

**Construction of pSIG and stable transfection**

Plasmid pGEM-T-SSTR2 bearing the full sequence of the SSTR2 gene (constructed by our laboratory, data not shown) was used as a template. SSTR2 was amplified using the sense (P1: 5′-GAAGATCT CTTATGGAAGAGGA GGAT-GTGAAGAGAGA GGTACCTGCGCCGTAGAG-3′) and the antisense (P2: 5′-AAAA CTGGCAGGTTT G2634 CCCAT TGCCA-GTAGACA-3′) primers. The PCR products were digested with appropriate restriction enzymes and then subcloned into plasmid pIRES2-EGFP (BD Biosciences Clontech, CA, USA) to generate pIRES2-EGFP (BD Biosciences Clontech, CA, USA) according to the manufacturer’s recommendations. G418 (Promega, Madison, WI, USA) resistant clones were expanded, harvested and named as MCF-7/pSIG cells. MCF-7 and MCF-7/pIRES2-EGFP cells were treated the same as mock and vector controls.

**SSTR2 mRNA expression assessed by quantitative real-time PCR**

Total RNA prepared from MCF-7, MDA-MB-231\(^\text{10}\), A549, HeplG2 and Hela cells were reverse-transcribed into their respective cDNAs. Equal amounts of cDNA were submitted to PCR, in the presence of SYBR green dye (Qiagen, Hilden, Germany) using the sense (P3: 5′-GGTGAACTGATTG-3′) primers. The β-actin endogenous gene (constructed by our laboratory, data not shown) was amplified using the sense (P3: 5′-GGTGAACTGATTG-3′) and the antisense (P4: 5′-G186AAGATGCT GGTGAACTGATTG-3′) primers. The β-actin endogenous housekeeping gene was used as an internal control. PCR without template was used as a negative control. Each sample was normalized by using the difference in critical thresholds (CT) between SSTR2 and β-actin, \(\Delta\Delta CT_{\text{SSTR2}}=\Delta CT_{\text{SSTR2}}-\Delta CT_{\text{β-actin}}\) where \(\Delta CT_{\text{SSTR2}}\) is the difference in CT between SSTR2 and the negative control, whereas \(\Delta CT_{\text{β-actin}}\) is between β-actin and the negative control. The mRNA levels of each sample were then compared using the expression \(2^{-\Delta\Delta CT_{\text{SSTR2}}}\). The expression level for the lowest one was arbitrarily assigned a value of 1, and the final results were expressed as fold numbers compared to the lowest sample.

The SSTR2 mRNA expression was also compared between various transfected MCF-7 cells by qRT-PCR.

**Flow cytometry analysis for SSTR2 expression**

Following incubation with 1:100 dilution of anti-SSTR2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; Catalog #, sc-11606) for 60 min, cells were cocultured with CY3-conjugated rabbit anti-goat IgG (Sigma, St Louis, MO, USA; Catalog #, C2821) for 60 min. After being washed and resuspended in PBS, cells were assessed with a flow cytometer (FCM, BD Biosciences, San Jose, CA, USA).

**Immunofluorescence assay for SSTR2 expression**

Paraformaldehyde-fixed MCF-7 cells were incubated with anti-SSTR2 polyclonal antibody overnight at 4°C. Following PBS washing, CY3-conjugated rabbit anti-goat IgG was added. Green and red fluorescence were viewed under a fluorescence microscope (Olympus, Tokyo, Japan). Negative controls were set the same as the FCM analysis.

**Internalization studies**

Studies of the internalization of somatostatin receptor-bound \(^{125}\text{I}-\text{labelled ligands (somatostatin analogue [D-Phe1, Tyr3] octreotide (TOC)) were performed. MCF-7 cells were incubated in triplicate with 150000 cpm \(^{125}\text{I}-\text{TOC at 37 °C. After 30, 60, 90, and 120 min, cells were rinsed twice. Surface bound radioactivity was removed by incubation with acid buffer (50 mmol/L glycine-HCl/100 mmol/L NaCl pH 2.8) at room temperature for 20 min. After two washing steps, the internalized activity was measured and related to the total activity added. After 3 washes, trypsin was added and the cells were transferred to scintillation vials for determination of bound radioactivity.**

**Apoptotic and cell cycle analyses**

MCF-7 cells were cultured with 1×10\(^{-9}\) mol/L TOC for 72 h. Then cells were fixed with 70% dehydrated alcohol overnight at 4 °C. Finally, cells were incubated in propidium iodide (PI, Sigma; Catalog #, 81845) for 20–40 min and assessed with FCM for the cell number in the sub-G\(_0\)/G\(_1\) peak (apoptotic peak) and for the DNA content.

**Detection for EGFR expression**

TOC treated cells were cocultured with PE-conjugated anti-EGFR (1:200 dilution, BD Biosciences; Catalog #, 555997). After 60 min of incubation, cells were assessed with FCM.

**MTT assay for EGF-stimulated proliferation**

2000 TOC treated cells were plated in a 96-well plate. Then cells were treated with epidermal growth factor (EGF, Peprotech EC, London, UK; Catalog #, AF-100-15) at different concentrations from 0 to 100 ng/mL. After 72 h, the MTT assay (MTT kit, Sigma; Catalog #, CGD1) was performed according to the protocol. \(OD\) values were read at 570 nm. Triplicates were set for each group.

**Results**

**Expression level of SSTR2 mRNA in different cancer cell lines**

An initial assessment of SSTR2 levels is essential for determining the response to the SST analog. The results of the qRT-PCR show that the expression of SSTR2 mRNA in the cancer cell lines varies considerably (Table 1). Among the several cancer cell lines, including the cells originating from neuroendocrine and non-neuroendocrine tumors, the SSTR2 mRNA was least expressed in the human breast adenocarcinoma...
Cells were plated into 24-well plates at a density of 0.5×10^5 cells/well and triplets were set. After total RNA was extracted, 1 μg RNA was reverse-transcribed. PCR was performed by 40 cycles of 5 s at 95 °C, 10 s at 60 °C and 10 s at 72 °C. Gene expression level was compared to that found in the lowest sample which was arbitrarily assigned the value 1. Δ∆CT data are means and standard deviations from three independent experiments.

Table 1. mRNA expression of SSTR2 on MCF-7, Hela, HepG2, MDA-MB-231, and A549 assessed by qRT-PCR.

| Cell lines   | Δ∆CT (Avg hSSTR2) | Normalized hSSTR2 amount relative to MCF-7 2^{−Δ∆CT hSSTR2} |
|--------------|-------------------|----------------------------------------------------------|
| MCF-7        | 13.237±0.594      | 1                                                        |
| Hela         | 8.343±0.506       | 29.733                                                   |
| HepG2        | 7.595±0.530       | 49.901                                                   |
| MDA-MB-231   | 9.752±0.788       | 11.197                                                   |
| A-549        | 9.448±0.436       | 13.823                                                   |

Table 2. Comparison of SSTR2 mRNA levels on MCF-7 cells by qRT-PCR.

| Cell group     | Δ∆CT (Avg hSSTR2) | Normalized hSSTR2 amount relative to MCF-7 2^{−Δ∆CT hSSTR2} |
|----------------|-------------------|----------------------------------------------------------|
| MCF-7          | 13.139±0.444      | 1                                                        |
| MCF-7/pIRES2-EGFP | 12.980±0.563   | 1.117                                                   |
| MCF-7/pSIG     | 1.724±0.720       | 2730.596                                                 |

The expression of EGFR in MCF-7 cells was lowered

EGFR expression clearly promotes growth and survival of tumor cells. EGFRs are as variably distributed in breast tumors and breast cancer cell lines as SSTRs[11]. To explore the relationship between the SSTR2 and EGFR, the EGFR levels of SSTR2-overexpressed MCF-7 cells were assessed. The results (Figure 4A) show that after treatment with TOC, the percent of EGFR-expressing cells in the MCF-7/pSIG group was lowered to 64.53%±10.05% (P<0.01 vs control). Additionally, there was also some reduction in the fluorescence intensity of the MCF-7/pSIG group, which suggests that the EGFR expression in MCF-7/pSIG cells was also decreased. However, the...
expression levels of EGFR in both controls remained high, at 93.01%±1.92% (vector control) and 92.14%±1.49% (mock control). Hence, the overexpression of SSTR2 appears to decrease the percentage of EGFR-expressing cells. Furthermore, SSTR2 overexpression was related to a reduction in the EGFR numbers/cell.

In support of biological relevance, the EGF-stimulated proliferation of MCF-7 cells at a physiological concentration was demonstrated by MTT assay. Figure 4B shows that, after treatment with EGF at different concentrations, more MCF-7 control cells exhibited proliferation than MCF-7/pSIG cells. This result indicates that the overexpression of SSTR2 could...
counteract the EGF-stimulated proliferation of MCF-7 cells. Notably, even without EGF stimulation (when the EGF concentration was 0 ng/mL), the MCF-7/pSIG cells proliferated less rapidly than the MCF-7 cells. This reconfirmed the anti-proliferative effects of SSTR2 in MCF-7 cells.

Cell cycle analysis (Figure 4C) shows that 5.61% of the MCF-7/pSIG cells were in S phase. That figure rose substantially to 32.52% and 22.36% in the untransfected cells and the vector control, respectively. These results show that SSTR2 may mediate the antiproliferative effect in MCF-7 cells.

Discussion
Many previous studies have indicated that SSTR expression may be able to give further insights into conventional prognostic and therapeutic approaches to breast cancer. Published reports have clearly demonstrated the antiproliferative action of SST and its analogs\cite{12, 13}. Tumor patients with poor prognoses are essentially characterized by a low expression of SSTRs\cite{14, 15}. In these patients, adjuvant treatment with SST might have little chance of success. Hence, poor expression of SSTR2 in breast cancer may result in the failure of hormonal therapy\cite{16}. In the present study, our data showed that at the mRNA level, SSTR2 was lowly expressed in the breast cancer cell lines MCF-7 and MDA-MB-231. Van Den Bossche et al\cite{16} also investigated the pattern of SSTR subtype expression in three other human breast cancer cell lines. They found that

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**Figure 3.** Apoptotic effect of SSTR2 on MCF-7 cells. Representative FCM photos showing the percentage of apoptotic cells. After being fixed with 70% dehydrated alcohol overnight, TOC-treated cells were stained with PI and then the cell numbers in the sub-G0/G1 peak were analyzed by FCM. The M1 gate demarcates the apoptotic cells. The percentage of gated cells is indicated. One representative experiment of three is presented.

**Figure 4.** Effect of SSTR2 on EGFR expression and function. (A) Representative FCM photos of the percentage of EGFR-expressing cells. Cells were cocultured with PE-conjugated anti-EGFR and assessed by FCM. One representative experiment of three is presented. (B) EGF-stimulated proliferation. Following treatment with EGF at different concentrations from 0 to 100 ng/mL for 72 h, cell proliferation was assessed by MTT assay. Data are means and standard deviations from three independent experiments. (C) Representative FCM photos demonstrating the relative percentages of cells in each phase of the cell cycle by PI staining. One representative experiment of three is presented.
SSTR2 was expressed at comparable levels in T47D, ZR75-1 and MDA-MB-231 cells. Hence, although the SSTR2 mRNA levels in T47D and ZR75-1 were not detected in this paper, given the data obtained from the MDA-MB-231 cells, we could suggest that the expression of SSTR2 appears to be significantly lower in breast cancer cell lines, especially in MCF-7 cells. Although the low expression of SSTR2 in MCF-7 cells was not confirmed by any protein assessment, the results are in accordance with previous reports\(^{[4, 9]}\).

Since SSTR2 is considered the principal mediator of the antiproliferative effects of SST, an inverse relationship between SSTR2 expression and tumor progression would be expected. In this research, a bicistronic plasmid, pSiG, expressing the human SSTR2 and EGFP genes separately, was constructed with an internal ribosome entry site (IRES)\(^{[17]}\). After confirmation of SSTR2 overexpression and SSTR2 binding affinities to the SST analogue TOC, the pSiG was stably transfected into breast adenocarcinoma MCF-7 cells. Our results demonstrated that when the expression was enhanced, SSTR2 exerted obvious antiproliferative effects on the MCF-7 cells, even when no TOC was administrated. A possible explanation could be that the breast cancer cell itself can secrete enough SST. However, whether SST can be synthesized and secreted from these tumor cells remains to be established. According to our study, the SSTR2 subtype may trigger apoptosis as well as cytostasis, although the mechanisms are not clear. To our knowledge, this is the first direct in vitro demonstration of the apoptotic effect of SSTR2 on the MCF-7 breast cancer cell line. Except for where it induces apoptosis, SSTR2 overexpression can also induce cell cycle arrest at the G\(_1\)/S phase interface.

The growth inhibition elicited by SST and its analogs can occur not only through interaction with the SSTRs present on a tumor cell membrane, but also through the inhibition of hormone and growth factor action\(^{[18, 19]}\). Increased levels of EGFR gene expression are observed in breast cancers, and they frequently seem to confer a poor prognosis\(^{[20]}\). Previous studies revealed the colocalization of SSTR and EGFR\(^{[9]}\) and the negative correlation between them\(^{[21]}\). Furthermore, the protein-protein interactions or dimerizations between ErbBs and SSTRs are important for signal transduction and are thought to be associated with tumor progression\(^{[5]}\). Here we investigated whether enhanced SSTR2 could participate in affecting the expression of EGFR and thereby counteract any deleterious effects of EGF. Although the mechanism remains unknown, our data validated this hypothesis and indirectly verified the crosstalk between the SSTRs and EGFR.

On the basis of the above observation, we concluded that the enhanced SSTR2 expression played an antiproliferative role in the MCF-7 cells through inducing apoptosis and G\(_1\)/S cell cycle arrest and also by possibly decreasing EGFR expression, thereby reversing the effects of EGF. Altogether, this suggests not only that a mechanism is needed to maintain or upregulate the SSTR2 level on the cell membrane in order to mediate the SST-induced antiproliferative effect, but also that SSTR2 needs to be activated in order to counteract the action of hormones and growth factors. Therefore, developing a new therapeutic agent that could activate SSTRs would potentially be a way to block tumor progression.

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**Author contribution**

Guan-xin SHEN and Yong HE designed research; Yong HE, Xiao-mei YUAN, Ping LEI, Sha WU, Wei XING and Xiao-li LAN performed research; Tao HUANG, Guo-bing WANG, Rui AN, Yong-xue ZHANG contributed new analytical tools and reagents; Xiao-mei YUAN and Hui-fen ZHU analyzed data; Yong HE, Xiao-mei YUAN and Ping LEI wrote the paper.

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