hSSTR2 expression and octreotide treatment reverses multidrug resistance of BxPC-3 human pancreatic cancer cells

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Abstract. Pancreatic cancer is generally refractory to most chemotherapeutic agents. We investigated whether hSSTR2 expression and octreotide treatment reverse multidrug resistance of human pancreatic cancer cells. We used pancreatic cancer cells that were transfected by using a lentivirus expression system, which allowed stable expression of the hSSTR2 gene in the pancreatic cancer cells. BxPC-3 cells were transfected with hSSTR2 through a lentivirus vector pWP XL-MOD-SSTR2 in order to enable the expression of hSSTR2. The transfected cells were treated with different concentrations of octreotide and with the chemotherapeutic agents cisplatin, epirubicin, fluorouracil and gemcitabine. The changes in IC_{50} following treatment with chemotherapeutic agents were determined, and the expression of different MDR indicating marker genes, multidrug resistance gene-1 (MDR1), multidrug resistance-associated protein 2 (MRP2), and lung resistance-related protein (LRP), were evaluated. Octreotide treatment of the transfected cells significantly decreased the IC_{50} of chemotherapeutic agents in a dose-dependent manner. hSSTR2 gene transfection decreased MDR1, MRP2 and LRP expression by 57, 47 and 56%, respectively ($P<0.01$), and octreotide treatment (1.6 μg/ml) for 48 h, decreased it further by 88, 73 and 87%, respectively ($P<0.01$). These data suggested that the down-regulation of MDR genes is responsible for the improvement in the chemotherapeutic sensitivity of hSSTR2-expressing pancreatic cancer cells, when these cells are subjected to octreotide treatment.

Introduction

Multiple drug resistance (MDR), a phenomenon wherein cells exposed to a single drug develop resistance to a broad range of structurally and functionally unrelated drugs, is a common cause of therapeutic ineffectiveness in cancers such as colon carcinomas, renal carcinomas, hepatomas, and pancreatic carcinomas (1). Most tumors contain a small percentage, approximately 2%, of MDR cells. Chemotherapy is not effective against these cells. After the first round of effective chemotherapy, all cells, except the MDR cells, are destroyed. Since non-MDR cells account for majority of the tumor mass, the tumor appears to be effectively destroyed. However, the MDR cells remain and start multiplying. If the cancer reoccurs due to MDR cells, the new tumor is likely to be entirely MDR. The next time chemotherapy is used, none of the cells will be destroyed. Pancreatic cancer cells, in particular, are resistant to both chemotherapy and radiation therapy. The exact mechanisms of resistance remain poorly understood. MDR is thought to be responsible for approximately 90% of chemotherapy failures. Hence, bypassing the MDR responses is a great challenge in the treatment of cancers (including pancreatic cancer). Due to limited efficacy and considerable toxicity of conventional chemotherapy, particularly in the case of pancreatic cancer, innovative non-cytotoxic approaches that can bypass MDR cells, are being developed. Amongst the various agents, we focused our attention on somatostatin (SST).

SST is a hormonal neuropeptide that exists in two active forms, namely, SST-14 and SST-28 with 14 and 28 amino acid residues, respectively. SST-14 and SST-28 interact with cells through a minimum of five membrane receptor subtypes that inhibit the secretion of various hormones including the growth hormone, which is also known as somatotropin (2). Studies have demonstrated that SST receptor (SSTRs) subtypes are strongly expressed in the normal pancreas, and even in the tissues adjacent to pancreatic cancers (3-5). In human pancreatic tumors, studies reveal that there is not only desensitization or mutation of these receptors in pancreatic adenocarcinomas and pancreatic endocrine tumors, but also a frequent loss of expression of these receptors, especially SSTR2 (6-8). SST and its analogs have demonstrated antineoplastic activities both *in vivo* and *in vitro*.

In this report, we demonstrated that human SSTR2 (hSSTR2), when introduced in the BxPC-3 cells can significantly lower the expression of the 3 MDR-associated genes such as multidrug resistance gene-1 (MDR1), multidrug resistance-associated protein 2 (MRP2), and lung resistance-

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related protein (LRP) (9-11). Octreotide is a synthetic octapeptide analog of endogenous SST having more potency and a longer half-life than SST. When transformed cells are subjected to octreotide treatment, the expression level of MDR marker genes further decreases. Since pancreatic cancer cells lack functional SSTRs, this re-introduction of hSSTR2 and subsequent decrease in the expression of genes involved in MDR indicates the possible role of hSSTR2 in MDR behavior. This was further supported by the fact that octreotide-treated cells showed a considerable decrease in the IC_{50} value following treatment with four different chemotherapeutic agents (epirubicin, 5-fluorouracil, cisplatin and gemcitabine). Gemcitabine and 5-fluorouracil are the most commonly used chemotherapy drugs against pancreatic cancer and epirubicin (anthracyclines) and cisplatin are the most commonly used chemotherapy drugs in other cancers.

Materials and methods

Cell culture. The human pancreatic cancer cell line BxPC-3 was purchased from the Shanghai Institute of Biological Products (Shanghai, China). BxPC-3 is a pancreatic cancer cell line, which can not produce SST even though the cells possess functional SSTRs on their surface (12). The cell line was maintained in 50% Dulbecco's Modified Eagle Medium (Gibco, USA) supplemented with 10% fetal calf serum (FCS, pH 7.2, Gibco). Cells were maintained at 37˚C in an atmosphere with 5% CO_2 and 100% humidity with a weekly serial passage. The medium was changed twice a week.

Construction of recombinant lentivirus vector pWP XL-MOD-hSSTR2. The hSSTR2 exon was amplified by polymerase chain reaction (PCR) using the hSSTR2-pCMV plasmid (Shanghai Telebio Biomedical, Inc., Shanghai, China) as a template. The following hSSTR2 primers were used for the amplification: upstream, 5'-GGGACGCGTATGGACAT GGCGGATGAG-3'; downstream, 5'-GCGACTAGTTCAGA TACTGGTTTGGAGG-3'.

The upstream and downstream primers have built-in Mlu I and Spe I restriction sites, respectively (hSSTR2-MluI/ hSSTR2-SpeI). The 1128-bp PCR product and the empty vector pWP XL-MOD (Shanghai Telebio Biomedical, Inc.) were double-digested with Mlu I and Spe I (New England Biolabs, Beverly, MA). The SSTR2 and pWP XL-MOD fragments were reclaimed using a gel extraction kit and linked with T_4 DNA ligase (New England Biolabs). After transformation into *Escherichia coli* DH5α, the positive clones were identified by restriction enzyme digestion and then sequenced for confirmation (comparator sequence was obtained from GenBank accession no. BC019610). The identical clones were named pWP XL-MOD-hSSTR2. In pWP XL-MOD-hSSTR2, the green fluorescent protein (GFP) and hSSTR2 genes were linked by the IRES, and the two genes were under the control of the same promoter, EFla.

Determination of pWP XL-MOD-hSSTR2 recombinant lentivirus titer. Using the Televector™ lentivirus vector system (Shanghai Telebio Biomedical, Inc.), 293T cells were transfected with the Recombinant plasmid pWP XL-MOD-hSSTR2 and three packaging system plasmids (pWP1, pWP2, pWP/VSVG). The supernatant from the culture medium was collected after 72 h, centrifuged at 1500 rotations per minute for 5 min to remove the cellular fragments, and filtered through a 0.45-μm cellular filter to obtain a crude extract of recombinant lentivirus containing the hSSTR2 and GFP genes. A crude extract of the empty-plasmid lentivirus containing only the GFP gene was obtained by an identical method. The crude extract was purified with a lentivirus purification kit in accordance with the instruction manual. The genomic ß-actin was tested and the virus was purified by fluorescent quantitative PCR. The lentiviral titer (Lv) was calculated by the following formula: Lv (Tu/ml) = (WPRE/ß-actin) x $5x10^3$ $x10^4$ x dilution multiple. The total RNA of the transfected 293T cells was extracted and identified by reverse transcription PCR (RT-PCR).

Determination of transfection efficacy of recombinant lentivirus. BxPC-3 cells were transfected with the recombinant lentivirus, multiplicity of infection (MIO)=10, to obtain the BxPC-3-GFP-hSSTR2 cell strain expressing hSSTR2 and GFP. BxPC-3 cells were also transfected with the empty-plasmid recombinant lentivirus (MIO=10) to obtain the BxPC-3-GFP cell strain expressing GFP. After 48 h of transfection with the recombinant lentivirus, the proportion of cells exhibiting green fluorescence was calculated with a fluorescence microscope.

Western blot analysis. We used BxPC-3 cells transfected with the recombinant lentivirus (MIO=10, 5, 3 and 2), with BxPC-3 cells transfected with the empty-plasmid recombinant lentivirus (MIO=10) acting as the negative control. After 48 h, the cells were subjected to protease inhibitor (1:200, Calbiochem, San Diego, CA, USA) and RIPA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) to obtain the total protein. Protein concentration was quantified by Bradford colorimetric determination. Based on the results, 100 μg of protein was electrophoresed. The primary antibodies were polyclonal goat anti-human SSTR2 goat polyclonal antibodies (1:400) and polyclonal goat anti-human ß-actin antibodies (1:500, Santa Cruz Biotechnology, Inc.). The secondary antibody was alkaline phosphatase-rabbit anti-goat antibosy (1:5000, Boster Biotechnology, Inc., Wuhan, China). Proteins on SDS-PAGE gels were transferred to nitrocellulose filters and stained. The bands were visualized by using an enhanced chemiluminescence system.

IC_{50} of chemotherapeutic agents followed by octreotide treatment at different concentration. BxPC-3-GFP-hSSTR2 cells in the exponential growth phase were trypsinized to yield a cell suspension of $5x10^4$ cells/ml and seeded onto 96-well plates ($0.5\text{-}1x10^4$ cells/well) in triplicate. The experimental group was treated with 0.4 or 1.6 μg/ml octreotide (OCT, Novartis, USA). While the blank group received the same volume of culture medium, the control group received the same volume of cell suspension. After the plates were incubated at 37˚C in 5% CO_2 for 48 h, 10 μl CCK-8 solution (Cell Counting Kit-8, Beyotime Institute of Biotechnology, China) was added to each well, the plates were reincubated for 1 h, and the optical density (OD) was read at an experimental wavelength of 540 nm and a reference wavelength of

Table I. Primer sequences and reaction condition in real-time PCR.

Gene	Primer	Reaction condition
Multidrug resistance gene-1(MDR1)	F: 5'-ggagtcattgtggagaaagg R: 5'-aaatgctggttgcaggcctc	40 cycles of 15 sec at 95˚C; 30 sec at 55˚C; 30 sec at 72˚C
Multidrug resistance-associated protein 2 (MRP2)	F: 5'-tgtcccacagcagtcctcggat R: 5'-catgagcatccactgcagacag	
Lung resistance-related protein (LRP)	F: 5'-catgagctggacgtgtgac R: 5'-agagccatctgcacagtcac	
Internal reference ß-actin	F: 5'-gcgagaagatgacccagctc R: 5'-ccagtggtacggccagcgg	

F, forward primer; R, reverse primer.

650 nm. Chemotherapeutic drugs were added after the cells were allowed to adhere for 24 h. The final concentrations of the chemotherapeutic drugs were as follows: epirubicin (EPI, Pfizer, USA), 1, 2, 4, 8 and 16 μg/ml; cisplatin (CDDP, Qilu Pharmaceutical, Inc., China), 2.5, 5, 10, 20 and 40 μg/ml; 5-fluorouracil (5-FU, Nantong General Pharmaceutical Factory, China), 12.5, 25, 50, 100 and 200 μg/ml; and gemcitabine (GEM; Lilly, USA), 0.00625, 0.0125, 0.025, 0.05 and 0.1 μg/ml.

The growth inhibition rates of the different drugs and concentrations were calculated by the following formula: Inhibition rate (fa) = (A1 value of negative control group - A value of experimental group)/(A1 value of negative control group - A0 value of blank group) x 100%. The IC_{50} value of each drug was calculated by Karber's method.

Relative quantitative analysis of MDR1, MRP2 and LRP genes. Using an RNA extraction kit (Invitrogen, Inc., USA), the total RNA was extracted from cell lines transfected with BxPC-3-GFP, BxPC-3-GFP-hSSTR2 and BxPC-3-GFP-hSSTR2 and treated with octreotide; this was followed by the chemotherapy. Total RNA (2 μg) from each set was reverse transcribed using reverse transcription kit (Promega, USA) to synthesize the cDNA.

The real-time PCR reaction system was as follows: 12.5 μl of 2X SYBR Green Mix (real-time PCR kit, Shanghai Shinegene Molecular Biotechnology, Inc., China), 1 μl of the forward and reverse primer (Table I) of target gene, 0.2 μl of cDNA. The total volume was made up to 25 μl. Analysis of relative gene expression data using $\Delta\Delta C_T$ method:

$$F=2^{-\Delta\Delta C_T}\ \Delta\Delta C_T = (C_{T,\,Target} - C_{T,\,Actin})_{Time\ x} - (C_{T,\,Target} - C_{T,\,Actin})_{Time\ 0}$$

Time x is any time-point and Time 0 repression of the target gene normalized to ß-actin (13).

Statistical analysis. Data are presented as mean ± SD. The data were analyzed by ANOVA with SPSS 13.0 (SPSS, Inc., Chicago, IL). A P-value <0.05 was considered statistically significant.

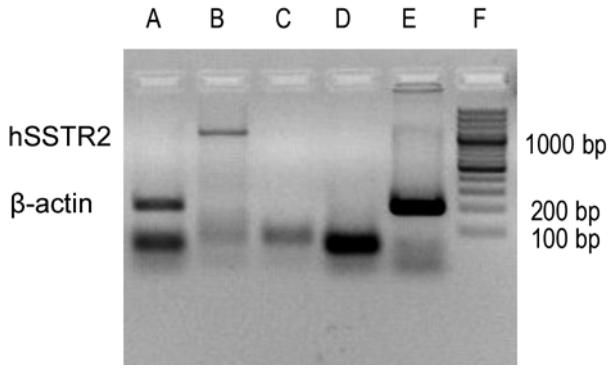

Figure 1. Identification of pWP XL-MOD-hSSTR2. pWP XL-MOD-hSSTR2 transfected 293T cells, the total RNA of the transfected 293T cells was reverse transcribed to obtain cDNA. The cDNA was further used as a template for amplifying the hSSTR2 gene (primers: hSSTR2-MluI/hSSTR2-SpeI), yielding a 1128-bp human SSTR2 cDNA fragment. Lane A, amplified cDNA of ß-actin (using ß-actin specific primers) from the cDNA amplified by RT-PCR from pWP XL-MOD-hSSTR2 transfected 293T cells (internal control), which serves the purpose of the control set; lane B, amplified cDNA of hSSTR2 from the same cells; lane C, amplified cDNA of hSSTR2 from pWP XL-MOD transfected cells; lanes D and E, the product after PCR amplification using SSTR2-specific primers (negative control) and ß-actin specific primers (internal control), from non-transfected 293 T cells; lane F, the marker.

Results

Confirmation of the pWP XL-MOD-hSSTR2 construct and confirmation of the transfection. The pWP XL-MOD-hSSTR2 clone was confirmed in multiple steps. The hSSTR2 sequence of pWP XL-MOD-hSSTR2 matched the normal hSSTR2 ORF sequence present in GenBank. The total RNA of the transfected 293T cells was reverse-transcripted to cDNA and used as a template for amplifying the hSSTR2 gene (primers: hSSTR2-MluI/hSSTR2-SpeI), yielding a 1128-bp hSSTR2 cDNA fragment (Fig. 1). The lentivirus titer was found to be $4.0x10^8$ TU/ml in the transfected cells. The transfection efficacy was monitored by transfecting the BxPC-3-GFP-hSSTR2 construct at a MOI of 10. Ninety percent of the cells

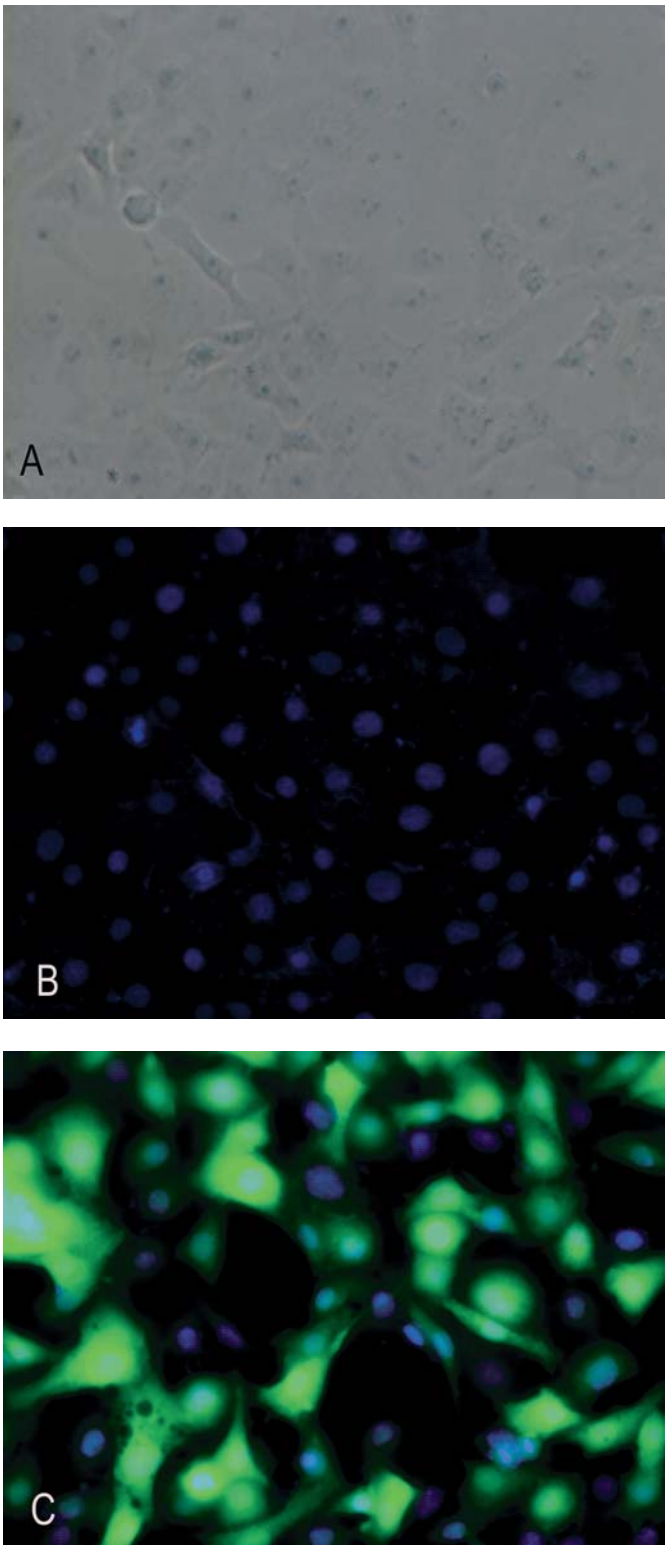

Figure 2. Analysis BxPC-3-GFP-hSSTR2 using fluorescence microscope. (A) Untransfected BxPC-3 cells (DAPI, under natural light). (B) Untransfected BxPC-3 cells (DAPI). (C) Transfected BxPC-3-GFP-hSSTR2 cells (DAPI).

showed fluorescence signals after 48 h of transfection as assessed by fluorescence microscopic analysis (Fig. 2).

hSSTR2 expression in BxPC-3 cells. An increase in the expression of hSSTR2 was observed with the increase in the MOI of infection. The relatively similar signal for ß-actin rules out the bias in the results owing to the presence of differential amount of transfected cells in the analyte (Fig. 3).

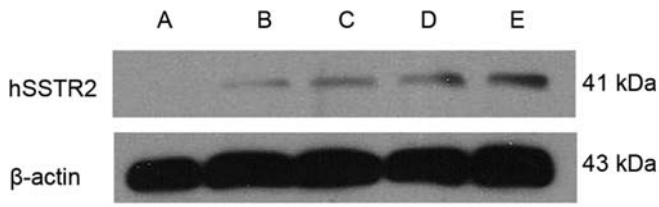

Figure 3. Western blot analysis of the hSSTR2 protein in the BxPC-3-GFP-hSSTR2 cell line at different MOI. Lane A, BxPC-3 cells infected by Lv-empty plasmid at MOI 5; lane B, BxPC-3 cells infected by Lv-hSSTR2 at MOI 2; lane C, BxPC-3 cell infected by Lv-hSSTR2 MOI 3; lane D, BxPC-3 cells infected by Lv-hSSTR2 MOI 5; lane E, BxPC-3 cells infected by Lv-SSTR2 MOI 10.

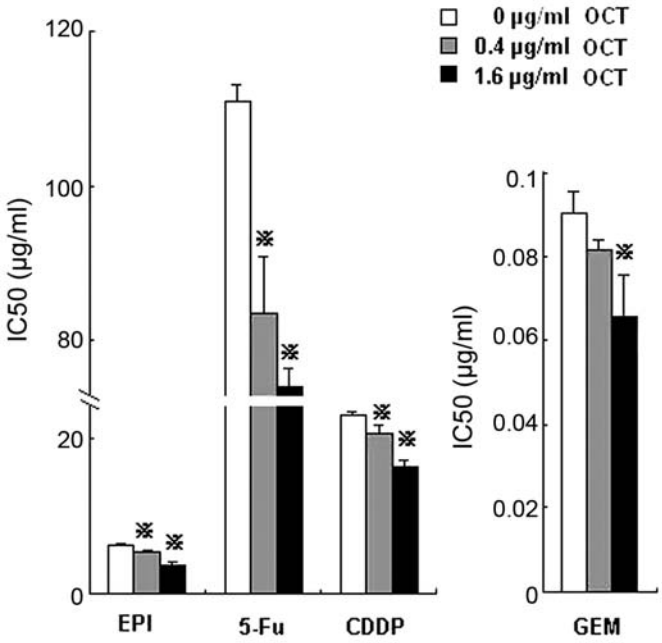

Figure 4. The IC_{50} of different chemotherapeutic drugs before and after treatment of the BxPC-3-GFP-hSSTR2 cells using different levels of octreotide. *P<0.05 (vs. 0 μg/ml OCT group).

IC_{50} of chemotherapeutic agents after octreotide treatment. The transfected cells were further treated with different concentrations of octreotide. This was followed by treatment with different chemotherapeutic agents. While the IC_{50} of the other chemotherapeutic agents showed a sharp decrease after octreotide treatment at all different concentrations (P<0.05), the IC_{50} of gemcitabine showed a noticeable decrease only with high-dose (1.6 μg/ml) octreotide treatment. The decrease in the IC_{50} values of the chemotherapeutic agents before and after octreotide treatment was statistically significant (P<0.05) (Table II, Fig. 4).

Expression of MDR1, MRP2 and LRP genes after hSSTR2 gene transfection and octreotide treatment. MDR1, MRP2 and LRP gene expression decreased by 57, 47 and 56%,

Table II. The IC_{50} of different chemotherapeutic drugs before and after treatment of the transfected cells using different levels (μg/ml, χ±s) of octreotide.

Octreotide	Epirubicin	5-Fluorouracil	Cisplatin	Gemcitabine
0	6.31±0.11	110.95±2.26	23.04±0.43	0.090±0.005
0.4	5.38±0.39[a]	83.75±7.22[a]	20.77±1.12[a]	0.082±0.002
1.6	3.70±0.33[a]	73.08±2.38[a]	16.30±1.04[a]	0.066±0.010[a]

[a]P<0.05 (vs. 0 μg/ml OCT group).

Table III. Expression of the MDR1, MRP2 and LRP genes in pancreatic cells before and after introduction of the SSTR2 gene and octreotide treatment.

	F-value		
Group	MDR1	MRP2	LRP
BxPC-3-GFP	1	1	1
BxPC-3-GFP-hSSTR2	0.43±0.015[a]	0.53±0.014[a]	0.44±0.015[a]
BxPC-3-GFP-hSSTR2+OCT	0.12±0.01[a,b]	0.27±0.013[a,b]	0.12±0.012[a,b]

[a]P<0.01 (vs. BxPC-3-GFP group), [b]P<0.01 (vs. BxPC-3-GFP-hSSTR2 group).

respectively only after the transfection. Hence, it can be concluded that SSTR2 expression alone can decrease the syndrome of drug resistance in pancreatic cancer cells. Furthermore, octreotide treatment (1.6 μg/ml) for 48 h further decreased the gene expression by 88, 73 and 87%, respectively (Table III).

Discussion

Several proteins can mediate MDR of tumor cells with different mechanisms of action. P-glycoprotein (P-gp, MDR1 gene product), an adenosine 5-triphosphate (ATP)-dependent membrane transporter and the recently discovered MDR associated protein 2 (MRP2), increase the cellular efflux of chemotherapeutic drugs (10) and cause MDR. Presence of MRP2 may correlate to intrinsic and acquired resistance for CDDP in human pancreatic cancer (14). Vault proteins, such as the lung resistance-related protein (LRP), which are involved in the drug transport from the nucleus to the cytoplasma, also play a major role in MDR (11). The LRP protein has been detected in human specimens of malignant melanomas with the highest expression in metastatic tissue exposed to chemotherapy (12). Hence, through several attempts, researchers have tried to identify the treatment of MDR in cancer patients that will reduce the expression of MDR1, MRP2 and LRP.

SST and somatostatin analogues (SSAs) exert their antiproliferative effect through SSTRs, especially SSTR2 (15-17). The synergy of SST and SSAs with chemotherapeutic agents can enhance the sensitivity and improve the curative effect of antineoplastic drugs (18,19). SSTR2 has been shown to suppress the growth of tumor cells in the absence of exogenous SST after the SSTR2 gene has been transfected into SSTR2-negative tumor cells (20-22). Most cancer cells that can be suppressed by SST and SSAs possess one or more superficial SSTRs. Also, SSTR2 is commonly associated with carcinomas (23-25). Published literature suggests that 90% human pancreatic cancer cells were found to lack SSTR2 mRNA and protein expression and are less sensitive to the SSAs (26).

Our results clearly demonstrate that octreotide significantly decreased the IC_{50} following treatment with the chemotherapeutic agents cisplatin, epirubicin, fluorouracil and gemcitabine in SSTR2-expressing pancreatic cancer cells in a dose-dependent manner. This indicates that SSTR2 expression probably helps the cells in overcoming the insensitivity of the pancreatic cancer cells to chemotherapeutic drugs. The reduction in the expression of the MRD indicative genes, i.e., MDR1, MRP2 and LRP following SSTR2 expression also supports this hypothesis. The further decrease in the expression of these genes following octreotide treatment indicates a new pathway for improving the sensitivity of pancreatic cancers to chemotherapy.

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