Expression of somatostatin and its receptor (SSTR) 1-5 gene in ectopic endormetrotic tissues and cells.

Hou Ping, Chen Long, Liu Hong, Zhang Weina, Liu Yansheng[*]

Department of Gynecology, Qingdao Municipal Hospital, PR China

Abstract

Objective: To detect the expression of somatostatin (SS) gene and somatostatin receptor (SSTR) 1-5 gene in endormetrotic tissues.

Methods: Real-Time PCR was applied to examine the expression of somatostatin gene in ectopic endometrial cells (EECs). The expression of somatostatin receptor 1-5 in ectopic endometrium, eutopic endometrium and normal endometrium and the relations with endometriosis (EMS) staging were determined by immunohistochemistry.

Results: The expression of SS gene in EECs was significantly higher compared with the control group. SSTR1-5 were expressed in the ectopic endormetrotic tissues from 30 patients with EMS, and the positive rates were 43.3%, 70%, 53.3%, 50% and 96.7%, respectively, which were not closely associated with EMS staging of the patients. The positive rates of SSTR1-5 expressions in the eutopic endometrium from 14 patients with EMS were 33.3%, 41.7%, 53.3%, 58.3% and 83.3%, respectively; while, the positive rates of SSTR1-5 expressions in the normal endometrium from 14 women without EMS were 7.1%, 7.1%, 21.4%, 28.6% and 64.3%, which were lower than the positive rates of SSTR1-5 both in the endormetrotic tissues and the eutopic endometrium.

Conclusions: SS gene was highly expressed in EECs. SSTR1-5 were expressed both in the ectopic and eutopic endometrium, the low or moderate expression of SSTR1-4 and the high expression of SSTR5 were detected in the ectopic and eutopic endometric tissues, and the low expression of SSTR1-4 and the partial expression of SSTR5 were detected in the normal endometrium. The positive rates of expression of SSTR1-5 in the endormetrotic and eutopic endometrium were higher than those in the normal endometrium. The expression of all the subtypes of SSTR in the ectopic endormetrotic tissues was not closely associated with EMS staging.

Keywords: Expression of somatostatin, Receptor (SSTR) 1-5 Gene, Ectopic endormetrotic tissues.

Accepted on December 06, 2017

Introduction

The incidence of EMS has increased in recent years, and become one of the most common gynecological diseases plaguing many women at childbearing age. The present treatment method for EMS mainly includes hormone therapy and surgical operation. The drug treatment effect is not ideal and the disease is easily recrudesce [1]. Somatostatin (SS) is a kind of peptide hormones with extensive physiological effects. Its physiological functions include hormone regulation, inhibition of cell proliferation, neurotransmitter release, inhibition of gastric acid, secretion of gastrin and pepsinogen, etc. A lot of studies showed that the somatostatin could inhibit the proliferation of tumor cells [2]. SS acts by binding to the special cell surface receptor (somatstatin receptor, SSTR). 5 kinds of SSTR subtypes named as SSTR1, SSTR2, SSTR3, SSTR4 and SSTR5. SSTR exists in a wide variety of neuroendocrine tumors and tumors in the nervous system [3-6]. Some studies have reported the expression of SSTR in endometrial cancer and ovarian cancer. However, rare studies

have focused on the expression of SSTR in EMS lesions. The present study is designed to detect the expression and significance of the expression of SS gene and SS receptor SSTR1-5 gene in the endometriotic tissue.

Materials and Methods

Tissue samples

The EMS tissues and cells were derived from 30 patients during gynecological laparotomy or laparoscopic operation in Xiangya Hospital from 2009 to 2016, including cystic walls from 28 cases with ovarian chocolate cyst; specimens from 2 patients with pelvic peritoneal EMS. Specimens were confirmed by Department of Pathology, Xiangya Hospital, fixed by 10% formalin and embedded with common paraffin. The patients had no special disease history, didn't treated with hormone therapy preoperatively within 3 months, were aged between 20 and 60 at an average (35.68 years old). The patinas were staged in line with The Revised American Fertility

Society (AFs) I: 2 patients with stage I, 7 cases stage II, 16 cases with stage III and 5 cases with stage IV. The eutopic endometrium were derived from 12 EMS patients undergoing the gynecologic abdominal hysterectomy or uterine laparoscopy from 2009 to 2016, all were endometrium at proliferative phase, and their average age was 40.52 years old. 14 specimens in the control group were collected from cervical CIN patients undergoing the surgical resection with normal endometrium from 2009 to 2016, all were endometrium at proliferative phase, their ages were 40~48 years old, and their average age was 45.18 years old.

Cell sources

Normal endometrium (NE) cells were obtained from healthy women undergoing curettage at childbearing age from 2012 to 2016 in outpatient department, Xiangya Hospital. The cctopic endometrial cells (EE) were obtained from the hospitalized patients undergoing the ovarian EMS cyst operation or laparoscopic surgery at the same time. The specimens were collected from 0.5-1mm-thick portion in inner cystic walls from ovarian chocolate cyst. The ages in both groups were 30.25+4.35 and 32.05+5.29 years old (P>0.05), all patients had no tumors, endocrine, immune and metabolic diseases and didn't administrate any hormone drug within 3 months. No pathological change was found in NE group by histological examination. The histological staging was consistent with the actual menstrual cycle; and ovarian EMS cyst was diagnosed in EE group.

Experimental materials

Goat anti-human SSTR1, SSTR2, SSTR3, SSTR4, SSTR5 polyclonal antibodies were purchased from Wuhan Boster Co., ltd; DMEM/F12 medium was purchased from Beijing Dingguo Biotechnology Co., Ltd; the fetal bovine serum was purchased from Shanghai Sijiqing Co., Ltd; and the collagenase IV and trypsin were purchased from Sigma Co., Ltd.

Cell separation and culture

By referring to modified Ryan et al. [7] and Overton et al. [8] methods: wash the obtained issues trice with PBS buffer, cut it into fragments about 0.5~1 mm^3, put in a 20 mL centrifugal tube, add 0.1% collagenase and 0.25% trypsin-digested solution by 2.5~5 times, mix evenly and water-bathe at 37°C and continuously oscillate to digest for 50~100 min in NE group and 80~120 min in EE group, add DMEM to stop digestion, repeatedly blow, filter the cells with 100-mesh stainless steel net, centrifuge the filtrate (mainly the mesenchymal cells and epithelial cells) at 1200 rpm for 10 min, remove the supernatant, add DMEM/F12 culture solution with 15% fetal bovine serum and blow the cell suspension, stain by trypan blue and count the cells, adjust the cell concentration, inoculate to the cell culture dish at 5×10^5/mL density, culture in an incubator with 5% CO_2 at 37°C, replace the medium once every 2~3 d till the cell convergence for primary culture. Directly observe the cell shapes and growth with inverted microscopy and photograph microscopically.

Real-time PCR

Primer design: With GAPDH gene as control, according to PCR primer design principle and human SST mRNA sequence in GeneBank, the SST and GAPDH gene primers were designed as below SST 138bp upstream GCTGCTGTCTGAACCC, downstream CGTTCTCGGGGGTGCCATAG, GAPDH 87bp upstream TGCACCACCAACTGC, downstream GGCATGGACTGTGGTCATGAG and the primers were synthesized by Nanjing GenScript Biotechnical Co., Ltd.

Reaction system:

DEPC H_2O 3.6 μL

10 uM primer F 1.2 μL

10 uM primer F 1.2 μL

10 uM primer R 1.2 μL

10 uM primer R 1.2 μL

SYBR® Green Realtime PCR Master Mix 10.0 μL

SYBR Green Realtime PCR Master Mix 10 μL

cDNA 2 μL

CDNA 2 μL

For totally 20 μL reaction system, each sample was prepared with 3 parallel wells and reacted on the eppendorf Master Realplex2 PCR at 95°C for 10 min; 95°C for 30 s; 58°C for 20S; 42 cycles; 72°C for 10 min. With the determination of ΔCt value, the relative expression level of target gene mRNA was indicated. Using GAPDH as an internal reference (ΔCt = target gene Ct value-HMBS Ct value), the relative changes in gene expression was indicated in $2^{-\Delta\Delta Ct}$ (ΔΔCt=ΔCt of sample group -ΔCt of control group).

Immunohistochemical method

The operation was done according to the instruction of immunohistochemical staining kit using the SABC approach. In the experiment, PBS instead of primary antibody was made as negative control and the known positive sections were used as positive control. The film was read by blind method under OLYMPUS microscope. Yellow or brown indicated the positive results. For the brown yellow particles in positive reaction of immunohistochemical staining, SSTR was mainly localized in the cytoplasm, the expression of SSTR was determined by using the quantitative method, the scores were summed up based on the coloring depth and the positive cell numbers, where 0 indicated positive staining, 1 light brown staining, 2 dark brown staining and 3 tan staining. 10 high magnification visionary fields were counted each to determine the positive rates of the each visionary field and calculate the average. The score (s) for positive cell number: 0 for<5%s, 1 for 5% to 24%, 2 for 25% to 49%, 3 for <50%~74% and 4 for >75%. The patients <1 score after both scores were multiplied were determined as negative in expression and the rest patients were determined as positive. 1~3 score (s) indicated weak

positive (+), 4-5 scores indicated positive (+), and greater than or equal to 6 scores indicated highly positive (+ +).

Statistical methods

With SPSS 19 software package for statistics, the expression of SSTR subtypes and clinical staging index were tested using χ^2 test and exact probability method and $P<0.05$ was deemed as significantly different.

Results

Real-time PCR

The relative expression histogram of SS genes in cells of EE and NE groups was automatically output by Stratagene Mx3000 supplied by Stratagene, USA.

Immunohistochemistry

Expression of SSTR subtypes in endometriotic tissues: The expression of SSTR was mostly located in cytoplasm and membrane in endometrial cells. In EMS cell from 30 cases, the positive expression of SSTR1 was from 13 patients (43.3%), the positive expression of SSTR2 was from 21 patients (70%), the positive expression of SSTR3 was from 16 patients (53.5%), the positive expression of SSTR4 was from 15 patients (50%), and the positive expression of SSTR5 was from 29 patients (96.7%). Compared with the positive expression from the control group, the expression rate of SSTR1, SSTR2, SSTR3 and SSTR5 increased significantly ($P<0.050$). The expression and clinical staging of SSTR subtypes were insignificantly related in endometriotic tissue (Table 1).

Table 1. The positive rate of expression and its relation to clinical staging of SSTR subtypes in endometriotic tissues.

			Clinical staging				P
		N	I	II	III	IV	
SSTR1	Positive	13	0	2	7	4	0.17
	Negative	17	2	5	11	1	
	Positive rate%	43.3					
SSTR2	Positive	21	1	5	11	5	0.418
	Negative	9	1	2	7	0	
	Positive rate%	70					
SSTR3	Positive	16	1	3	9	3	0.936
	Negative	14	1	4	7	2	
	Positive rate%	53.3					
SSTR4	Positive	15	0	3	10	4	0.233
	Negative	15	2	4	6	1	
	Positive rate%	50					
SSTR5	Positive	29	2	6	16	5	0.467
	Negative	1	0	1	0	0	
	Positive rate%	96.7					

Expression of SSTR subtypes of in eutopic endometrium tissues in EMS and normal control group: The positive expression rates of SSTR1-5 from 14 cases with eutopic endometrium in EMS tissues were 33.3%, 41.7%, 53.3%, 58.3% and 83.3% , and the positive expression rate of SSTR2 was significantly higher than the control group ($P<0.05$); the positive expression rates of SSTR1-5 in normal endometrium was lower in the ectopic and eutopic endometrium groups at 7.1%, 7.1%, 21.4%, 28.6% and 64.3% respectively (Table 2) .

Table 2. Expression of SSTR subtypes in endometrial tissues of ectopic, eutopic and control endometrium groups.

	n	-	+-	+	++	Positive rate %	P
Control	14						
SSTR1		13	1	0	0	7.1	
SSTR2		13	1	0	0	7.1	
SSTR3		11	2	1	0	21.4	
SSTR4		10	2	2	0	28.6	
SSTR5		6	4	3	1	64.3	
Eutopic endometrium	12						
SSTR1		8	2	2	0	33.3	0.091
SSTR2		7	3	2	0	41.7	0.037 *
SSTR3		5	4	2	1	58.3	0.054
SSTR4		5	5	1	1	58.3	0.126
SSTR5		2	4	4	2	83.3	0.155
Ectopic endometrium	30						
SSTR1		17	6	4	3	43.3	0.016 *
SSTR2		9	11	6	4	70	0.000 *
SSTR3		14	7	6	3	53.3	0.047 *
SSTR4		15	8	5	2	50	0.181
SSTR5		1	6	14	9	96.7	0.002 *

Discussion

SS is a kind of natural polypeptide hormone containing 14 or 28 amino acids, and occurs widely in human endocrine and exocrine systems and has extensive biological, mainly inhibitory, effects. The physiological functions of SS include hormone regulation, inhibition of cell proliferation, neurotransmitter release, inhibition of gastric acid, secretion of

pepsin and gastrin, reduction of splanchnic blood flow, also inhibition of release of insulin like growth factor -1 (IGF-1) , platelet-derived growth factor (PDGF) , epidermal growth factor (EGF) , and white cell interleukin -6 (IL-6) and interferon-γ (IFN-γ) and other cytokines. SS clinical application is restricted as a result of natural SS in a very short half-life *in vivo*, 4 min only. The somatostatinanalogue (SSTA) has the advantages of long half-life, relatively long-lasting effect, high selectivity and easy use etc. The somatostatin and its analogues have been used to treat neurological diseases, such as Alzheimer's disease, chorea, epilepsy; tumors, such as pituitary tumor, pancreatic islet cell tumor, carcinoid tumor; and some gastrointestinal diseases, such as gastric ulcer, stomach bleeding and digestive tract ulcer, acute pancreatitis, bleeding from esophageal varices etc. at home and abroad [1-5]. SS is extensively distributed in the digestive system and nervous system. Many cancer cells, inflammatory cells and immune cells (lymphocytes, phagocytic cells, such as endothelial cells, thymus etc.) produce SS as well [6]. The growth hormone, insulin-like growth factor, IL 1, IL 6, TNF-a, IFN-γ and other growth factors and cytokines was well as glucocorticoid, androgen and estrogen can increase the expression of SS gene whilst insulin and leptin can inhibit its expression. The intracellular factors regulating the expression of SS gene mainly include cAMP, Ca^{2+}, cGMP and NO [6]. The cAMP plays a promoting function on expression and secretion of SS gene, thus becomes a signal transduction pathway that regulates the SS function. The cAMP is mainly induced through phosphorylation of cAMP-dependent protein kinase A to regulate the transcription of SS gene [6].

EMS is an estrogen-dependent ill. The estrogen can stimulate the growth of endometriotic cells. The Real-time PCR results in the present study showed that the expression of SS gene in endometriotic cells was higher than in normal endometrial cells significantly, suggesting that the estrogen is closely associated to the expression and regulation of and SS gene. The previous studies have showed that the occurrence of EMS is related to the immune mechanism. The intraperitoneal mononuclear macrophage in EMS patients increases in the activity, secretes a large level of inflammatory mediators, such as interleukin (IL) 1, 6 and TNF-a, etc, and then creates the conditions for the EMS proliferation [7]. These factors can promote the expression of SS gene. The present study showed that the high expression of SS gene in EMS cells suggested that the increased expression of SS gene in EMS may be likely related to its occurrence and development mechanism.

Many studies showed that SS can be used as a kind of important hormone regulatory peptide for cell proliferation and differentiation to inhibit the proliferation and angiogenesis of tumor cells [8]. SS inhibition mechanism mainly includes direct inhibition and indirect effect. SS, SSTA and SSTR after binding and activation can play an ant-proliferative role thought 4 major signal pathways [3]: l) The cAMP pathway to changes cAMP metabolism. The cAMP decrease can inhibit cell proliferation and affect protein synthesis [6]; 2) Intracellular Ca2+ change to reduce the membrane permeability against Ca^{2+}, inhibit Ca^{2+} influx, block the Ca^{2+}-

mediated intracellular reaction [5]; 3) The phosphate protein kinase pathways: after the SSTR binds to ligands, the tyrosine kinase is inactivated after dephosphorylation to inhibit protein kinases, such as MAPK, and so inhibit the cell proliferation [9,10]; 4) The phosphatidylinositol-3-kinase pathway: SSTA inhibits the proliferation of tumor cells through the inhibition of phosphatidylinositol-3-kinase [11]. SSTA's indirect inhibition of activates Fas, Caspase-8 and MAPK pathways over receptors mediation, blocks cell transformation from G1 phase to S phase, inhibits the cell proliferation and promote apoptosis [12,13]. SSTA's indirect inhibition also presents in the inhibition of angiogenesis [14,15]. Although EMS is histologically benign, but has wide malignant behaviors, such as planting, infiltration, metastasis and relapse, transferability to abdominal wall, intestinal tract and genitourinary tract to cause the periodic pain and symptoms; the ectopic endometrium as autografts rely more on vascular support and the angiogenesis is the key to the development of EMS. The occurrence and development of EMS may be inhibited through the above mechanism by using SSTAK. The estimate needs to be further verified.

SS acts through binding to specific receptors on cell surface. SS receptor is a membrane protein receptor, and divided into 5 subtypes. Due to 7 transmembrane domains, it is known as the SSTR. According to the similarity and varying affinity towards SSTA, SS can be divided two types: 1) high affinity to SSTA, including SSTR2, SSTR3, SSTR5 and SSTA; 2) weaker affinity, including SSTRl, SSTR4. SSTRl, SSTR2 and SSTR5 mainly mediating the anti-proliferative effect. SSTR is distributed in all the major human lymphoid organs, includingthymus, spleen, lymph nodes, tonsil and gut associated lymphoid tissues [16]. SSTR occurs in a wide variety of neuroendocrine tumors and tumors of the nervous system. But SSTR also expressed in non-neuroendocrine tumor tissues, for example, SSTR were positive for over 50% breast cancer, 40% colorectal cancer [17,18]; it is also expressed in liver, prostate, pancreatic cancers and gastric MALT lymphoma and its metastases [19-22]. Schulz et al has detected the expression of SSTRl, SSTR2 and SSTR3 in 12 patients with cervical carcinoma at the positive rates 38%, 57% and 43% respectively, the expression of SSTR4 and SSTR5 was negative and the SSTR1-5 was expressed in 18 patients with endometrial carcinoma. The positive rates were 32%, 39%, 43%, 4% and 4% respectively [23]. Expression and positioning of SSTR vary from human tumor tissues. SSTR expresses different subtypes in different lesion tissue, and the expression level of similar subtypes is different, and the SST and its analogues have different binding forces and play different roles.

Although a large number of studies have been conducted for SSTA and SSTR in other systems, rare researches have focused on the expression of SSTR in endometriotic lesion tissues. Green et al have researched the expression of SSTR2 in the endometrium during the human menstrual cycle using the immunohistochemistry and real-time quantitative PCR method. The results showed that SSTR2 was expressed throughout the menstrual cycle [24]. Fasciani et al. discovered that SSTRl,

SSTR2 and SSTR5 were highly expressed in the ovarian and peritoneal endometrial lesions using immunohistochemistry and real-time quantitative PCR method. In a cell experiment, it was found that the octreotide can inhibit the migration and proliferation of endometrial cells [25]. Recently, Annunziata et al. found the SSTR1-5 was expressed in endometriotic tissues and cells, SSTR1 was mainly expressed, STR3 and SSTR2 were lowly expressed and SSTR4 and SSTR5 were not expressed in normal eutopic endometrial cells [26].

Immunohistochemical results in the present study showed that SSTR1-5 was expressed in EMS tissues from 30 patients at the positive rates 43.3%, 70%, 53.3%, 50% and 96.7% respectively, which was not significantly associated to the clinical stage of patients and consistent with what found by Fasciani and Annunziata et al. The positive expression rate of SSTR1-5 in eutopic endometrium from 14 EMS patients were 33.3%, 41.7%, 53.3%, 58.3% and 83.3% respectively whist the positive expression rate of SSTR1-5 in normal endometrial tissue were lower than in ectopic and eutopic endometrium groups at 7.1%, 7.1%, 21.4%, 28.6% and 64.3% respectively. The results verified that SSTR1-5 was expressed in ectopic and eutopic endometrium groups and thus provides a therapeutic basis for the EMS using SSTA.

The results of the present study showed that the positive expression rate of SSTR1-5 in endometriotic and eutopic endometrium cells was higher than in normal endometrium, suggesting that the similarity between eutopic and endometriotic cells and difference between it with normal endometrium, further confirming the "eutopic endometrium determinism" theory. Therefore, the detection of SSTR expression in the eutopic endometrium is clinically significant. As many as clinical studies confirmed that the SSTA, such as octreotide, can play an inhibitory role on pancreatic cancer, breast cancer, neuroendocrine tumors and carcinoid tumor [3-5]. In recent years, a series of non-peptide agonist L-797591, L-779976 and L-803087 acting together with 5 SSTR receptors were developed. Their affinity towards the receptors is hundreds of times the peptide agonists. These SSTAs with high affinity explore a broad foreground for clinical application. SSTR with high affinity and low side effect may bring happiness and chance to the EMS patients that are positive to SSTR.

The scintigraphy (somatostatin receptor imaging, Somatstain Receptor Scintigraphy, SRS) with radiolabeled SSTA has been used for locating diagnosis of tumor. The radiolabeled SST analogues can bind to the SSTR-positive tumors and help diagnosis definitely. 111In-DTPA octreotide has been used in conventional clinical examination for SSTR-positive neuroendocrine tumor. Fasciani et al. injected 111In-DTPA octreotide on the 8 EMS patients preoperatively, scanned after 12 h, found the stronger signals in pelvic cavity from 6 patients and weaker signal in pelvic cavity from 2 patients and didn't found any adverse reaction after examination [25]. Although EMS is benign histologically, it has wide malignant behaviors, such as planting, infiltration, metastasis and relapse. By scintigraphy using the radiolabeled SSTA, EMS can be located

and diagnosed and the endometriotic lesions that B ultrasound and CT cannot detect in pelvic cavity and intestinal duct can be discovered and the auxiliary diagnosis can be made for the EMS-induced chronic pelvic pains.

Some studies have shown that the expression level of SSTR can be improved by transfection of SSTR gene for some cancer cells that the SSTR is lowly or not expressed in some tissues, such as pancreatic cancer, gastric cancer, colorectal cancer and ovarian cancer, thus improving the efficacy of SSTA [27,28]. For the endometrial cells that the expression level of somatostatin receptor is low, the somatostatin receptor with high expression level can be genetically induced, thus providing likelihood for the diagnosis and treatment of EMS using the SS analogs. The present study found that the SS and its receptor are positively expressed in EMS tissues or cells and provided an experimental basis for the diagnosis and treatment of EMS using the SSTR gene transfection and SSTA approaches.

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*Correspondence to

Liu Yansheng

Department of Gynecology

Qingdao Municipal Hospital

PR China