COLORECTAL CANCER

Relationship between somatostatin receptor subtype expression and clinicopathology, Ki-67, Bcl-2 and p53 in colorectal cancer

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Received: 2005-09-23 Accepted: 2005-11-18

Abstract

AIM: To study the SSTR1, 2, 3, 4, 5 expression and their relationships with clinico-pathological factors, cell proliferation, Bcl-2 and p53 expression in colorectal cancer cells.

METHODS: Immunohistochemical staining of five SSTR subtypes, Ki-67, Bcl-2 and p53 was performed by the standard streptavidin-peroxidase (SP) technique for the paraffin sections of 127 colorectal cancers, and expression of five SSTR subtypes in 40 specimens of normal colorectal mucosa was detected with the same method.

RESULTS: Positive staining for five SSTR subtypes was observed in colorectal cancer cells and normal colorectal mucosa. SSTR1 was the most predominant subtype in both colorectal cancer and normal colorectal mucosa, and the second was SSTR5 or SSTR2. As compared with normal colorectal mucosa, SSTR4 was more frequently expressed in colorectal cancer cells (2.5% vs 18.9%, P<0.05); the expression of SSTR2, 4, 5 in moderately to well differentiated colorectal adenocarcinoma was significantly higher than that in poorly differentiated ones (P<0.05), the SSTR1 expression in colorectal cancer with positive lymph node metastasis was significantly higher than that with negative lymph node metastasis (72.2% and 54.5%, P<0.05). In addition, in the ulcerative type of colorectal cancer, SSTR2 expression was obviously decreased (P<0.05); the correlation did not reach a statistical significance between the five SSTR subtypes expression and Dukes' stages (P>0.05), but the frequency of SSTR1 expression increased with Dukes' stage, while SSTR3 and SSTR5 expression decreased with Dukes' stage. Moreover, there was no correlation between expression of the five SSTR subtypes and other clinicopathological factors such as age, sex, tumor site, tumor depth, distant metastasis. The proliferative indexes in colorectal cancer cells with negative expression of SSTR2 and SSTR3 were significantly higher than that with positive expression (P<0.05). The Bcl-2 expression in colorectal cancer cells with positive expression of SSTR1, 2, 3, 5 was significantly lower than that with negative expression (P<0.05). There was no correlation between five SSTR subtypes and p53 expression.

CONCLUSION: The most predominant SSTR subtype is SSTR1, and the second is SSTR2 or SSTR5. Five SSTR subtypes play different roles in the development of colorectal cancer. SSTR2 and SSTR3 can inhibit the proliferation and promote apoptosis of tumor cells.

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Key words: Somatostatin receptor subtype; Cell proliferation; Apoptosis; p53; Colorectal cancer; Immunohistochemistry

Qiu CZ, Wang C, Huang ZX, Zhu SZ, Wu YY, Qiu JL. Relationship between somatostatin receptor subtype expression and clinicopathology, Ki-67, Bcl-2 and p53 in colorectal cancer. World J Gastroenterol 2006; 12(13): 2011-2015

http://www.wjgnet.com/1007-9327/12/2011.asp

INTRODUCTION

Somatostatin analogue (SSTA) may inhibit the growth of various tumor cells by direct interaction with specific somatostatin receptors (SSTR) on tumor cells. However, conflicting clinical results were obtained in patients with colorectal cancers treated with SSTA[1]. Many authors think the rationale for the clinical efficacy of SSTA in the management of colorectal cancer may be related to the direct inhibitory effect of SSTA, consequently, with the expression of SSTR subtypes on tumor cells[2,3]. To date, five SSTR subtypes, SSTR1-SSTR5, have been cloned in human tissues, however, few researches on their expres-
cancer were reported, and the results were inconsistent. In this study, we used an immunohistochemical method to detect the expression of five SSTR subtypes protein in colorectal cancer cells and to explore correlation between each subtype expression and clinicopathological factors, cell proliferation or apoptosis. It will provide the basis for the treatment of colorectal cancer with SSTA.

MATERIALS AND METHODS

Patients and samples
A total of 127 cases of colorectal adenocarcinoma were involved in this study. The patients with colorectal carcinomas, who underwent surgical resection at the Second Hospital Affiliated to Fujian Medical University (Quanzhou, China) from January 1, 2003 to June 30, 2004, had received neither chemotherapy nor radiation therapy before surgery. There were 73 men and 54 women, and their mean age was 58.5 (SD, 13.9; range, 24–84) years. specimens were obtained from right colon (31), left colon (27), rectum (69). According to the Chinese Dukes’ Staging criteria, 15 cases were stage A, 31 cases were stage B, 46 cases were stage C and 14 cases were stage D. Among 127 patients, 102 were moderately to well differentiated and 25 poorly differentiated adenocarcinomas. Meantime, 40 specimens of morphologically normal colorectal mucosas were examined from the same patients at a minimal distance of 10 cm from the diseased area.

All specimens were routinely fixed in neutral-buffered formalin and embedded in paraffin. Serial 4 μm sections were cut from archived paraffin blocks and subjected to immunohistochemical study. In each case, standard hematoxylin-eosin staining was employed for routine histological examination.

Reagents

Goat anti-human SSTR2, SSTR3, SSTR4 and SSTR5 polyclonal antibody were purchased from Santa Cruz Biotechnology Co. (California, U.S.A.). Rabbit anti-human SSTR1 polyclonal antibody, mouse anti-human Ki-67 monoclonal antibody (MBL1), mouse anti-human Bcl-2 monoclonal antibody (100/D5), mouse anti-human p53 monoclonal antibody (DO-7), SP staining kit and DAB kit were supplied by Maixin-Bio Co., Fuzhou, China.

Methods

The immunohistochemical staining for five SSTR subtypes, Ki-67, Bcl-2 and p53 expression in colorectal cancer cells and five SSTR subtypes expression in normal colorectal mucosas were carried out by the standard streptavidin-peroxidase (SP) technique. Briefly, after deparaffinization and rehydration, the antigen retrieval of the sections was achieved by incubation in 0.01mol/L citric acid buffer (pH 6.0) and boiled for 1 min in a pressure cooker, and then cooled and washed in tap water. The sections were incubated with hydrogen peroxide for 10 min and washed in PBS. Nonspecific reactions were blocked by incubating the sections in a solution containing normal serum. The sections were incubated with a primary antibody (anti-SSTR1, SSTR2, SSTR3, SSTR4, SSTR5, Ki-67, Bcl-2 or p53 antibodies) overnight at 4°C. The working dilution of anti-SSTR2, SSTR3, SSTR4, SSTR5 antibodies was 1:400. Rinsed with PBS, then the sections were incubated for 10 min at room temperature with biotinylated secondary antibody. After washing, streptavidin biotin complex conjugated to horse-radish peroxidase was applied for 10 min at room temperature. Again after three rinses with PBS, the sections were incubated with diaminobenzidine substrate, then rinsed with distilled water and counterstained with hematoxylin, finally dehydrated and cover slipped. The sections were prepared for microscopic observation. The known positive colorectal cancer tissues were used as positive control. As negative control, PBS was used to replace primary antibody.

Scoring criteria for SSTR subtypes expression

Intensity and percentage of positive cells were used to evaluate each tissue section. The mean percentage of positive tumor cells to normal epithelial cells in at least five areas at 400 magnification was determined and assigned to one of five categories: 1) 0, < 5%; 2) 1, 5% to 24%; 3) 2, 25% to 49%; 4) 3, 50% to 74%; and 5) 4, ≥ 75%. The intensity of SSTR subtype immunostaining was scored as 0 (achromatic), 1(light yellow), 2(yellow), and 3(brown). The percentage of positive cells and staining intensity were multiplied to produce a weighted score for each case. Cases with weighted scores less than 1 were defined as negative; all others were defined as positive.

Evaluation of staining for p53 and Bcl-2

For p53 or Bcl-2 expression, cases with ≤ 10% positively-stained tumor cells were defined as negative; otherwise, the definition was positive.

Determination of the Ki-67 proliferative index

At least 5 high-power fields were chosen randomly in each section, and 500 cells were counted for each field. The Ki-67 proliferative index was defined as the number of Ki-67-positive nuclei divided by the total number of colorectal cancer cells counted and was expressed as a percentage.

Statistical analysis

The statistical software package SPSS 11.5 was used. Clinicopathological variables associated with SSTR subtypes expression as well as the correlation between SSTR subtypes and p53 or Bcl-2 expression were analyzed by either the χ² test or Fisher’s exact test. The correlation between SSTR subtypes and proliferative index was analyzed by r test. P<0.05 was considered significant.

RESULTS

Expression of five SSTR subtypes

Positive-staining for all SSTR subtypes was identified in the cytoplasm and membrane of colorectal cancer cells and normal colorectal mucosas with brown-yellow granules (Figures 1-5). The positive rates of SSTR1, SSTR2, SSTR3, SSTR4 and SSTR5 expression were respectively 64.6% (52/127), 36.2% (46/127), 18.9% (24/127), 18.9% (24/127), 38.6% (49/127) in colorectal cancer and 52.5% (21/40), 40% (16/40), 30% (12/40), 25% (10/40), 32.5% (13/40) in normal colorectal mucosas (Table 1). SSTR1 was
the most predominant subtype in both colorectal cancer and normal colorectal mucosa, and the second was SSTR5 or SSTR2. As compared with normal colorectal mucosa, SSTR4 was more frequently expressed in colorectal cancer cells (2.5% vs 18.9%, P < 0.05), and the positive rates of other subtypes expression were not different between colorectal cancer and normal colorectal mucosa (P > 0.05).

### Correlation between SSTR expression and clinicopathological factors

The expression of SSTR2, 4, 5 in moderately to well differentiated colorectal adenocarcinoma was significantly higher than that in poorly differentiated colorectal adenocarcinoma (P < 0.05)(Table 2). The SSTR1 expression in colorectal cancer with positive lymph node metastasis was significantly higher than that with negative lymph node metastasis (72.2% and 54.5%, P < 0.05). The correlation did not reach a statistical significance between the five SSTR subtypes expression and Dukes’ stages (P > 0.05), but the frequency of SSTR1 expression increased with Dukes’ stage, while SSTR3 and SSTR5 decreased with Dukes’ stage. In addition, in the ulcerative type of colorectal cancer, SSTR2 expression was obviously decreased (P < 0.05); however, there was no correlation between expression of the five SSTR subtypes and other clinicopathological factors such as age, sex, tumor site, tumor depth, distant metastasis.

### Correlation between SSTR expression and proliferative index and Bcl-2 or p53

The proliferative indexes in colorectal cancer cells with negative expression of SSTR2 and SSTR3 were significantly higher than that with positive expression (P < 0.05, Table 3). A positive cytoplasmic immunoreactivity for Bcl-2 was detected in 67 of 127 cases (52.8%). The Bcl-2 expression in colorectal cancer cells with positive expression of SSTR1, 2, 3, 5 were significantly lower than that with negative expression (P < 0.05, Table 3). In contrast, nuclear accumulation of p53 was demonstrated in 81 of 127 cases (63.8%). There was no correlation between five SSTR subtypes and p53 expression.

### DISCUSSION

Five SSTR subtypes belong to G proteins family, and different SSTR subtypes can be expressed in various patterns in different normal tissues or tumors. Evaluation of five SSTR subtypes expression in tumors may help understand carcinogenesis, and progression of tumors, as well as diagnosis and treatment. There are some controversies on the dominant SSTR subtype and the correlation between five SSTR subtypes expression and clinicopathological factors in colorectal cancer cells. Buscail et al found that SSTR2 expression was lost in colorectal cancer [3]. However, some subsequent studies demonstrated that there was no difference between SSTR subtypes expression between normal colorectal mucosae and colorectal cancer[5-7]. Also some results indicated the most frequent subtype was SSTR2 or SSTR5 in colorectal cancer [5,8]. The expression of SSTR2 or SSTR5 was significantly lower in Dukes’ C and D stages of colorectal cancer than that in Dukes’ A and B stages. The decrease of SSTR2 or SSTR5 expression may be related to tumor local invasion and metastasis, which may provide a growth advantage in colorectal cancer. It is possible that SSTR2 or SSTR5 acts as a tumor suppressor[9,10]. But recent research showed there was no correlation of SSTR mRNA expression with Dukes’ stages[3].

In this study, only the frequency of SSTR4 expression in colorectal cancer cells was higher than that in the colorectal mucosae. SSTR1 was expressed predominantly in colorectal cancer cells, followed by SSTR5 and SSTR2. There was no correlation between five subtypes expression and Dukes’ stages. The differences of our results from previous findings may be accounted for by different research methods. SSTR subtypes can express not only in colorectal cancer cells but also in other constituents of cancer tissue, such as vessels[9]. Thus, the PCR method tends to overestimate the real contribution of the various messengers for receptors owing to the outstandingly sensitive technique, and mRNA levels do not necessarily reflect the presence of the SSTR protein in certain cancers. To detect protein expression of SSTR by receptor autoradiography can not completely reflect protein levels of five SSTR subtypes, because there are major differences in binding affinity of SSTA to various SSTR subtypes[10]. In addition, the discrepancy may be relevant to different reagents and the small sample size of our study. Cascade reaction induced by activation of SSTR2 and SSTR5 can inhibit proliferation of tumor cells. In our study, the expression of SSTR2, SSTR4 and SSTR5 reduced in poorly differentiated colorectal cancer, and the SSTR3 and SSTR5 expression showed a tendency to reduction with Dukes’ stages. The loss of these receptors can weaken inhibition on the proliferation of tumor cells, and may provide a growth advantage in colorectal cancer. It suggests SSTR2, 3, 4, 5 may be related to the development of colorectal cancer.

This study also demonstrated that the Ki-67 proliferative index in colorectal cancer cells with positive expression of SSTR2 or SSTR3 was significantly lower than that with negative expression of SSTR2 or SSTR3. This further proved that somatostatin in vivo can directly inhibit colorectal cancer cell proliferation by activating SSTR2 and SSTR3. The transplanted models of pancreatic primary tumor and hepatic metastases were established in hamsters which were xenografted with human pancreatic cancer cell line transferred with the SSTR2 gene, and the following effects were induced: growth of tumors with SSTR2 positive expression was delayed and the Ki-67 proliferative index was decreased significantly[10,11]. Activation of SSTR2 and

| Table 1 SSTR expression in colorectal cancer |
|---------------------------------------------|
| Tissue | n | SSTR1 | Expression | n (%) | SSTR2 | SSTR3 | SSTR4 | SSTR5 |
|--------|---|-------|------------|-------|-------|-------|-------|-------|
| Normal mucosa | 40 | 21(52.5) | 16(40.0) | 12(30.0) | 1(2.5) | 13(32.5) |
| Colorectal cancer | 127 | 52(46.4) | 46(36.2) | 24(18.9) | 24(18.9) | 40(38.6) |

*P*<0.05 vs normal mucosa.
Ki67 proliferation  
Expression of  
Expression of  
mean±SD  positive (%)  positive (%)  

| Clinico-pathological factor | n SSTR1 | SSTR2 | SSTR3 | SSTR4 | SSTR5 |
|-----------------------------|---------|-------|-------|-------|-------|
| Sex                         |         |       |       |       |       |
| Male                        | 73      | 47(64.4) | 26(35.6) | 13(17.8) | 13(17.8) | 28(38.4) |
| Female                      | 54      | 35(64.8) | 20(37.0) | 11(20.4) | 11(20.4) | 21(38.9) |
| Age (yr)                    |         |       |       |       |       |
| < 60                        | 66      | 49(68.2) | 20(30.3) | 12(18.2) | 12(18.2) | 24(36.4) |
| ≥ 60                        | 61      | 37(60.7) | 26(42.6) | 12(19.7) | 12(19.7) | 25(41.0) |
| Tumor site                  |         |       |       |       |       |
| Right colon                 | 31      | 18(58.1) | 11(35.5) | 7(22.6) | 6(19.4) | 11(35.5) |
| Left colon                  | 27      | 17(63.0) | 12(44.4) | 5(18.5) | 4(14.8) | 13(48.1) |
| Rectum                      | 69      | 42(61.1) | 28(39.2) | 16(23.0) | 12(17.4) | 21(36.6) |
| Macrosopic type             |         |       |       |       |       |
| Elevated                    | 40      | 27(67.5) | 17(42.5) | 18(45.0) | 9(22.5) | 17(42.5) |
| Ulcerative                  | 59      | 37(62.7) | 13(22.0) | 8(13.6) | 8(13.6) | 21(35.6) |
| Infiltrative                | 28      | 16(57.1) | 9(32.1) | 6(21.4) | 7(25.0) | 11(39.3) |
| Differentiation             |         |       |       |       |       |
| Moderate                   | 102     | 70(68.6) | 43(42.2) | 22(21.6) | 24(23.5) | 46(45.1) |
| Poor                       | 25      | 12(48.0) | 3(12.0) | 2(8.0) | 0(0.0) | 3(12.0) |
| Tumor depth                 |         |       |       |       |       |
| Muscularis                  | 24      | 14(58.3) | 11(45.8) | 5(20.8) | 3(12.5) | 9(37.5) |
| Serum                      | 103     | 68(66.0) | 35(34.0) | 19(18.4) | 21(20.4) | 40(38.8) |
| Lymph node metastasis       | (+)     | 72      | 52(72.2) | 24(33.3) | 12(16.7) | 15(20.8) | 26(36.1) |
| (-)                        | 55      | 30(54.5) | 22(40.0) | 12(21.8) | 9(16.4) | 23(41.8) |
| Distant metastasis          | (+)     | 20      | 15(75.0) | 6(30.0) | 3(15.0) | 2(10.0) | 7(35.0) |
| (-)                        | 107     | 67(62.6) | 40(37.4) | 21(19.6) | 22(20.6) | 42(39.3) |
| Dukes’ stage               | A       | 13      | 7(53.8) | 5(38.5) | 4(30.8) | 2(15.4) | 6(46.2) |
| B                           | 40      | 22(55.0) | 16(40.0) | 8(20.0) | 7(17.5) | 16(40.0) |
| C                           | 54      | 36(70.4) | 19(35.2) | 9(16.7) | 13(24.1) | 20(37.0) |
| D                           | 20      | 15(75.0) | 6(30.0) | 3(15.0) | 2(10.0) | 7(35.0) |

*P < 0.05.

| Clinico-pathological factor | n SSTR1 | SSTR2 | SSTR3 | SSTR4 | SSTR5 |
|-----------------------------|---------|-------|-------|-------|-------|
| Kid67 proliferation index   | mean±SD | Expression of anti-apoptosis Bcl-2 positive (%) | Expression of anti-apoptosis p53 positive (%) |       |       |
| SSTR1 (+)                   | 82      | 34.22±24.33 | 37.45(1.5) | 53(64.6) |       |       |
| (-)                         | 45      | 40.21±22.97 | 30(66.7) | 28(62.2) |       |       |
| SSTR2 (+)                   | 46      | 28.65±18.80 | 15(32.6) | 33(71.7) |       |       |
| (-)                         | 81      | 40.71±25.90 | 52(64.2) | 48(51.3) |       |       |
| SSTR3 (+)                   | 24      | 25.72±17.88 | 5(20.8) | 17(70.8) |       |       |
| (-)                         | 103     | 38.82±24.55 | 62(60.2) | 64(62.1) |       |       |
| SSTR4 (+)                   | 24      | 28.02±20.12 | 9(37.5) | 16(66.7) |       |       |
| (-)                         | 103     | 38.29±24.42 | 58(56.3) | 65(63.1) |       |       |
| SSTR5 (+)                   | 49      | 32.20±21.92 | 17(34.7) | 34(69.4) |       |       |
| (-)                         | 78      | 38.95±24.91 | 50(64.1) | 47(60.3) |       |       |

*P < 0.05.

The activation of some SSTR subtypes not only directly inhibits the proliferation of tumor cells, but also is relevant to apoptosis. Bcl-2 is an apoptosis suppressor gene and important parameter of apoptosis. In this study, Bcl-2 expression in colorectal cancer cells with positive expression of SSTR1, SSTR2, SSTR3 or SSTR5 significantly decreased, indicating the four SSTR subtypes can promote the apoptosis of tumor cells and affect the progression of colorectal cancer by down-regulating the Bcl-2 expression or counteracting the anti-apoptosis effect of Bcl-2. They promote apoptosis via different mechanisms: Apoptosis is mediated by SSTR1 via a block in the G1/S progression in the cell cycle[14]. SSTR2 activation can promote apoptosis through the following pathways, it sensitizes cancer cells to apoptosis induced by tumor necrosis factor, induces caspase-8 activation cascade, stimulates mitochondrial cytochrome c released into the cytosol and downregulates the Bcl-2 expression[15]. In the mouse models of pancreatic primary tumor and hepatic metastases, which were xenografted with human pancreatic cancer cell line transferred with the SSTR2 gene, it has been demonstrated that activation of caspase-3 and apoptotic index increased, expression of anti-apoptosis protein Bcl-2 decreased in tumor cells expressing SSTR2[14,16]. Apoptosis can be signaled by SSTR3 which can activate phosphoprotein phosphates and lead to cellular acidification and activation of acidic endonuclease[17].

In conclusion, five SSTR subtypes play different roles in the development of colorectal cancer. SSTR2 and SSTR3 can inhibit the proliferation and promote apoptosis of tumor cells. The expression of SSTR2, SSTR4 and SSTR5 is correlated with malignant degree of colorectal cancer cells. Though SSTR1 is the predominant SSTR subtype, its ex-
pression is correlated with lymph node metastasis. SST1, which has a high affinity to SSTR2, SSTR3 and/or SSTR5 should be a treatment choice for patients with colorectal carcinomas.

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S- Editor Pan BR  L- Editor Zhu LH  E- Editor Wu M