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Research of the relationship between β-catenin and c-myc-mediated Wnt pathway and laterally spreading tumors occurrence

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Abstract. – OBJECTIVE: We studied the relationship between the occurrence of laterally spreading tumors (LSTs) and the activation of Wnt/β-catenin/c-myc pathway.

PATIENTS AND METHODS: 20 cases of colorectal polypoid adenomas (PAs) tissue sample, 20 cases of LSTs tissue sample and 20 cases of colorectal cancer tissue sample were acquired, and positive expression of Wnt/β-catenin/c-myc proteins were detected by immunohistochemical staining. In addition, relative expression levels of mRNA and protein were measured using RT-PCR and Western blotting method.

RESULTS: The positive expression levels of Wnt/β-catenin/c-myc protein in the colorectal cancer group were higher than that in LST group, and the value of the PAs group was the lowest; differences were statistically significant ($p < 0.05$). The relative expression levels of Wnt/β-catenin/c-myc mRNA and protein in colorectal cancer group were higher than that in LST group, and the value of PAs group was the lowest; differences were statistically significant ($p < 0.05$).

CONCLUSIONS: The activation level of the Wnt/β-catenin/c-myc pathway has a close relation to the occurrence and deterioration of LSTs.

Key Words:
Laterally spreading tumors, Wnt/β-catenin/c-myc, Polypoid adenomas, Colorectal cancer.

Introduction

It was confirmed that the colorectal adenoma polyp is the main precancerous lesion of colorectal cancer, and laterally spreading tumors (LSTs) are a special form of colorectal adenoma polyps[1]. They manifest as flat extrusion of the colorectal mucosa, growing laterally along the surface of intestine wall mucosa, with shallow infiltration, which usually requires a chromo endoscopy as an auxiliary examination[2]. According to statistics[3], the detection rate of LSTs are about 0.5-2.0%. Adenoma and mucous cancer demonstrate the main pathological pattern with diameters larger than 10 mm and have a higher malignance potential compared to polypoid adenomas (PAs), rectum and cecum as the common occurrence part[4]. The occurrence and development of colorectal cancer are mostly due to multi-factors and multi-elements involvement and gradual change, among which the Wnt cell signaling transduction pathway is closely related to the occurrence of colorectal cancer[5,6]. As a cancer suppressor gene, adenomatous polyposis coli (APC) gene plays an important role in the early cancerization of most colorectal cancer. β-catenin protein is a key molecule in the Wnt signaling pathway system, but also a main factor of Wnt signaling pathway activation in the endonuclease[7-9]. C-myc and cyclinD1 are the main downstream target genes of the Wnt signaling pathway, which can condition division cycle, proliferation, apoptosis and other biological behaviors of cancer cells. Based on that, this study aims to analyze the relationship between the occurrence of LSTs and the activation of Wnt/β-catenin/c-myc pathway.

Patients and Methods

Patients

20 patients with Pas, 20 patients with LSTs and 20 patients with colorectal cancer that were admitted to our hospital from Jan. 2013 to Jan. 2016 were selected successively, without previous operation and chemo radiotherapy history. The exclusion criteria included inflammatory bowel disease and familial adenomatous polypo-

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sis. The focus tissue samples were acquired using an endoscope or by excision. Baseline information of three groups was comparable (Table I).

Methods

Positive expression of Wnt/β-catenin/c-myc protein was detected by immunohistochemical staining. In addition, relative expression levels of mRNA and protein were measured by RT-PCR and the Western blot method. Section making: conventional methods include embedding, fixing and section, with a thickness of 8 µm, and under -80°C to preserve.

Immunohistochemical Staining

Sectioning was conducted with lost-wax, and hydrogen dioxide solution was added to inactivate the endogenous peroxidase. For antigen retrieval, sheep serum was used to seal the antigen, and anti-mouse Wnt, β-catenin and c-myc monoclonal antibody (Sigma-Aldrich Co., St. Louis, MO, USA, working concentration of 1:2000) was added under 4°C to stay overnight with a rewarming and PBS washing of 5 min × 3 times. An anti-mouse second antibody IgG (Jiangsu Beyotime Science and Technology Ltd., Shangai, China, working concentration of 1:2000) was added, which was incubated at 27°C for 1 hour. Phosphate Buffered Saline (PBS) washing was conducted for 5 min × 3 times and placed in an immunohistochemical wet box (Invitrogen Co., Carlsbad, CA, USA) to react for 1 hour with PBS washing of 5 min × 3 times. Diaminobenzidine (DAB) coloration, hematoxylin staining, dehydration of gradient ethanol, xylene transparency, neutral resins seal sheet and the slides were observed under microscope in order to take images. The known positive section was taken as a positive control, PBS as a negative control, yellow to dark brown in the cytoplasm or karyon as the positive standard. Positive cell proportion and color intensity were calculated, among which non-yellow was recorded as 0 score, faint yellow as 1 score, intermediate yellow as 2 scores, and brown yellow as 3 scores. The microscope with 400 times magnification was selected. The five sections included left, right, up, down and center, and 200 cells were observed at each section. The positive cell proportion less than 10% was marked as 0 score, 10-50% as 1 score, 50-75% as 2 scores, ≥ 75% as 3 scores. The accumulated scores of two items were multiplied to get the total score. The 0 score as negative, 1-3 scores as positive, 4-7 as moderately positive, 7 as strongly positive.

RT-PCR Methods

The RNA was extracted using a conventional TRIzol method, and the concentration and purity of RNA was detected by Ultraviolet Spectrometry Photometer, with a RT-PCR Kit compound cDNA and primer designed synthesis by Takara, Shiga, Otsu, Japan. Wnt (F): 5'-TACCTCCAGTCACACTCCCC-3', (R): 5'-CCATGGCAGGAGAATAGGA-3', 348 bp; β-catenin (F): 5'-TGCAGTTCGCCTTCACTATG-3', (R): 5'-ACTAGTCGTGGAATGGCACC-3', 162 bp; c-myc (F): 5'-ATGCCCCTCAACGTTAGC-3', (R): 5'-AGCTCGCTCTGCTGCTGC-3', 143 bp; internal control GAPDH9 (F): 5'-TGCTTCACCACCTTCTTGA-3', (R): 5'-TCACCATCTTCCAGGAGC-3', 456 bp. With a reaction system of 5 × buffer 2.5 µl + MgCl2 1.5 µl + dNTP 0.5 µl + GAP-43 and 2 µl internal control upstream and downstream primer 1 µl + Taq enzyme 0.3 µl + cDNA template, water was added until the total volume reached 25 µl. Reaction condition of 95°C for 5 min, 5°C for 30 s, 62°C for 30s and 72°C for 30s repeated 35 times, and 72°C for 10 min. The solubility curve was created, and the relative expression quantity of mRNA was calculated by $2^{-\Delta\Delta Ct}$ methods.

Western Blot Methods

Protein was extracted using a RIPA lysis solution, reacting for 30 min on ice, with acute oscillation for 1 min with an interval of 5 min. The

Table I. Comparison of baseline information of three groups.

Groups	Pas (n = 20)	LSTs (n = 20)	Colorectal cancer (n = 20)	F/χ^2	p
M/F	12/8	11/9	10/10	0.404	0.817
Age (y)	52.6 ± 13.2	53.4 ± 14.5	55.2 ± 13.3	0.315	0.862
Maximum Diameter (mm)	9.5 ± 2.6	12.2 ± 3.4	11.5 ± 3.5	0.625	0.537
Rectum	13	10	11	0.950	0.622
Colon	7	10	9		

sample was placed under 4°C and centrifuged at 2000 g for 20 min. The supernatant as the sample holoprotein; protein concentation was measured by the Bradford method (Invitrogen Co., Carlsbad, CA, USA) with SDS-PAGE gel electrophoresis (50V for spacer gel, 100V for separation gel, and an electrophoresis time of 3 hours). The PVDF transfer membrane, 5% skim milk powder confining liquid was added and incubated at room temperature for 4 hours. The attenuation anti-goat Wnt, β-catenin and c-myc monoclonal antibody (Zhongshan Golden Bridge Biotechnology Co., Ltd, Beijing, China, working concentration of 1:2500) and internal control β-actin (Zhongshan Golden Bridge Biotechnology Co., Ltd, Beijing, China, working concentration of 1:2500) were added and incubated at 37°C for 1 hour, with TBST washes for 5 min × 3 times. The rabbit anti-Goat secondary antibody IgG (R&D Co., Minneapolis, MN, USA, working concentration of 1:200) was added and incubated at 37°C for 1 hour. The TBST washes occurred for 5 min × 3 times. The ECL color, scanning, and image analysis software IPP6.0 (Media Cybernetics Co., Rockville, MD, USA) were applied to procced with gray intensity analysis.

Statistical Analysis

The Software SPSS20.0 (SPSS Inc., Chicago, IL, USA) was used to analyze the results statistically. The measurement data was presented by mean ± standard deviation. The comparison between the two groups took the single factor ANOVA to analyze, and the pairwise comparison applied LSD-t method to detect. Enumeration data was shown by the number of cases or (%), and comparison among many groups applied χ^2-test to detect. The ranked data used rank sum test. $p < 0.05$ meant the difference had statistical significance.

Results

Comparison of Immunohistochemical Staining Results

The positive expression levels of Wnt/β-catenin/c-myc protein in colorectal cancer group was higher than that in the LST group, and the value of the PA group was the lowest; differences were statistically significant ($p < 0.05$) (Table II).

Comparison of RT-PCR Methods Results

The relative expression levels of Wnt/β-catenin/c-myc mRNA in colorectal cancer group was higher than that in the LSTs group, and the value of the PAs group was the lowest; differences were statistically significant ($p < 0.05$) (Table III).

Comparison of Western Blot Methods Results

The relative expression levels of Wnt/β-catenin/c-myc protein in colorectal cancer groups were higher than those in the LST group, and the value of the PAs group was the lowest; differences were statistically significant ($p < 0.05$) (Table IV).

Discussion

Previous studies have verified that k-ras, p53 and cyclooxygenase (COX-2) are closely associated with the occurrence of LSTs and colorectal cancer. The mutation rate of k-ras in LSTs cancerization reaches 70-80%, which is significantly higher than the value of 20-30% of PAs[10]. p53 is related to the cancer inhibition effects of many malignant cancers[11-13]. COX-2 is the key rate-limiting enzyme of prostaglandin synthetics, which have the ability to inhibit apoptosis and

Table II. Comparison of immunohistochemical staining results [cases (%)].

Groups		Pas (n = 20)	LSTs (n = 20)	Colorectal cancer (n = 20)	Z	p
Wnt	Negative	15 (75.0)	6 (30.0)	3 (15.0)	17.417	0.002
	Positive-Moderately Positive	3 (15.0)	8 (40.0)	7 (35.0)		
	Strongly Positive	2 (10.0)	6 (30.0)	10 (50.0)		
β-catenin	Negative	13 (65.0)	5 (25.0)	4 (20.0)	11.557	0.021
	Positive-Moderately Positive	4 (20.0)	10 (50.0)	8 (40.0)		
	Strongly Positive	3 (15.0)	5 (25.0)	8 (40.0)		
c-myc	Negative	12 (60.0)	5 (25.0)	4 (20.0)	10.048	0.040
	Positive-Moderately Positive	6 (30.0)	8 (40.0)	7 (35.0)		
	Strongly Positive	2 (10.0)	7 (35.0)	9 (45.0)		

Table III. Comparison of RT-PCR methods results.

Groups	Pas (n = 20)	LSTs (n = 20)	Colorectal cancer (n = 20)	F	p
Wnt	0.1235 ± 0.0639	0.3629 ± 0.1235	0.5214 ± 0.1527	9.632	0.000
β-catenin	0.1026 ± 0.0528	0.3725 ± 0.1027	0.5328 ± 0.1634	8.629	0.000
c-myc	0.1324 ± 0.0467	0.3638 ± 0.1163	0.5166 ± 0.1529	10.325	0.000

promote cell adhesion; they are highly expressed in most gastroenteric tumors, and are related to many factors such as occurrence of tumor, pathological stage and prognosis[14]. The LSTs cell line (LST-R1), created in China, has been confirmed to have a high degree of malignant potential by ultrastructure, chromosome and heterogeneity animal inoculation experiments[15]. Through the study of various adherence factors such as E-cadherin, TM4SF9 and galectin-1 in LST-R1, we found that there is a lack of adherence factor expression in cells that might be related to the special form of lateral, superficial and spreading growth[16].

From this research, we concluded that the positive expression levels of Wnt/β-catenin/c-myc proteins in the colorectal cancer group were higher than that in the LSTs group, and the value of the PAs group is the lowest; differences were statistically significant. The relative expression levels of Wnt/β-catenin/c-myc mRNA and protein in the colorectal cancer group were higher than those in the LSTs group, and the value of PA group was the lowest; differences were statistically significant. These results suggest that the activation level of Wnt/β-catenin/c-myc pathway is closely related to the occurrence and deterioration of LSTs. 90% or above colorectal cancer has the activation of the Wnt typical signaling pathway. Therefore, it has become a new research direction to cure colorectal cancer, and taking the key molecules in this signaling pathway as a target spot, with β-catenin as positive regulatory factor, and APC, Axin, glycogen synthase kinase (GSK) -3β and casein kinase (CK) 1 as negative regulatory factors[17]. In the molecular structure of β-catenin, amino terminal contains several phosphorylation sites of GSK-3β and carboxyl terminal, which has the function of activating relevant target gene transcription, forming a -helix and go-between structure in the intermediate region, integrating with Cadherin, APC protein and intranuclear transcription factor T cell factor (TCF). Therefore, the stability and accumulation of β-catenin within cells is the key to Wnt signaling pathway activation[18]. C-myc protein is an important regulatory factor for cell proliferation, which was considered to be the progression factor of stage G1[19]. C-myc can also induce cell apoptosis, whose regulatory direction depends on the signal received by cells and the growth environment that cells are placed in[20]. Some research shows that C-myc can activate the telomerase of cells to induce its transcription and “immortalize” cells, therefore, promoting the formation of a tumor[21]. Furthermore, C-myc can also facilitate the expression of vascular endothelial growth factor (VEGF), as well as aid in the formation of new blood vessels in tumor[22]. The cancer gene C-myc possesses the function of “molecular switch” in gene transcription and cell regulation, which is activated during the evolution process from colorectal adenoma to adenocarcinoma, leading to C-myc with abnormal activation, increased protein expression, and promoting cell proliferation[23].

In addition, the DNA methylation state of multiple key genes that regulate Wnt also has a close relation to the occurrence colorectal cancer. In addition, DNA methylation has a re-

Table IV. Comparison of Western blot methods results.

Groups	Pas (n = 20)	LSTs (n = 20)	Colorectal cancer (n = 20)	F	p
Wnt	0.12 ± 0.05	0.35 ± 0.12	0.58 ± 0.19	7.628	0.000
β-catenin	0.13 ± 0.06	0.34 ± 0.11	0.61 ± 0.21	8.324	0.000
c-myc	0.11 ± 0.04	0.36 ± 0.14	0.63 ± 0.18	8.629	0.000

versible gene modification, which may become an efficient target spot of molecule intervention. Nosho et al[24] show that CpG island hypermethylation of LST tissue DNA is closely related to B-raf gene mutation. Hashimoto et al[25] demonstrated that hypermethylation of the APC gene has an inverse correlation to the mucous invasion of LST.

Conclusions

We observed that the activation level of Wnt/β-catenin/c-myc pathway has a close relation to the occurrence and deterioration of LSTs.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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