Expression and clinical significance of Sirt1 in colorectal cancer

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Abstract. The objective of the present study was to examine the expression of Silent information regulator 1 (Sirt1) in colorectal cancer and peritumoral normal mucosa tissue, and therefore analyze the role and molecular mechanism of Sirt1 in the pathogenesis of colorectal cancer. Colorectal cancer tissue specimens were employed as the experimental group, and adjacent normal mucosa tissues >5 cm from tumor lesions were used as the control group. The expression of Sirt1 was detected by the immunohistochemical streptavidin peroxidase detection method in paraffin-embedded sections, whilst Sirt1 protein expression was examined by western blot analysis in the fresh tissues. Sirt1 protein was primarily expressed in the nuclei of the tumor cells, and positive staining was brownish-yellow in color. The relative expression quantities of Sirt1 in the peritumoral normal rectal mucosa and rectal carcinoma were 1.15 and 2.62, and the differences between the two groups were statistically significant (P<0.05). The expression level of Sirt1 in colorectal carcinoma was significantly associated with the depth of tumor invasion, differentiation and tumor size (P<0.05). Sirt1 expression was also found to be associated with tumor tissue type, lymph node metastasis, Duke's stage and patient age. These characteristics combined may therefore be used as markers for the early diagnosis of colorectal cancer pathogenesis.

Introduction

Colorectal cancer occurs in the colorectal mucosa and colonic glands and is one of the most common types of malignant tumor of the digestive system (1,2). The worldwide incidence and mortality rates of colorectal cancer increased significantly

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between 2009 and 2013; in 2013, a total of 142,820 new cases were diagnosed and 50,830 mortalities occurred as a result of colorectal cancer (3). Thus, this malignancy presents a serious threat to human health (1,2). Clinical and pathological data of patients with colorectal cancer in China have demonstrated the following pathogenic characteristics: The number of patients in cities is higher than that in the countryside, indicating a clear urbanization trend; and patients <30 years of age account for >10% of the total number of patients, demonstrating a clear trend towards older age (4). Surgical resection is currently the main method used for the treatment of colorectal cancer. However, as significant symptoms are often not present in the early stages of the disease, patients are frequently diagnosed in the later stages; by this time, the optimal period for surgery has passed. Furthermore, metastasis or recurrence occurs in a large number of patients following surgical resection, affecting the prognosis of the patients. Therefore, the five-year survival rate is low (64%), posing a serious threat to patient health (5,6).

The occurrence and pathogenesis of colorectal cancer is a complex, multistep process, regulated by a number of different genes (1). A total of 25% of patients with colorectal cancer have a genetic history, which is associated with familial adenomatous polyposis and hereditary non-polyposis colorectal cancer (7). Results of molecular pathology and colorectal cancer expression profile chip screening have demonstrated that multiple genes serve regulatory roles in the processes of colorectal cancer development (8). In order to identify possible gene target therapies and individualized treatments, various studies have focused on genes associated with the pathogenesis of colorectal cancer and their mechanisms. Previous studies have employed various methods to explore potential markers for the early diagnosis and prognosis of colorectal cancer; however, at present, its primary cause is unknown, and therefore further research is required (9).

Recent studies have demonstrated that Silent information regulator 1 (Sirt1) can regulate the deacetylation of lysine residues of multiple proteins, which is dependent upon nicotinamide adenine dinucleotide (NAD$^+$). Sirt1, a member of the Sirtuin family, is a type of class III histone deacetylase (10). The Sirtuin family regulates gene expression and is involved in the regulation of various biological events in cells. In particular, Sirt1 is important in cell survival, senescence, apoptosis, differentiation and other metabolic processes (11). Studies

have demonstrated that the expression of Sirt1 is increased in prostate cancer (12) and acute myelocytic leukemia (13). Hida *et al* (14) observed that the expression of Sirt1 was also significantly enhanced in a variety of types of non-melanoma skin cancer, including squamous cell carcinoma, basal cell carcinoma, Bowen's disease and actinic keratosis. The NAD$^+$-dependent deacetylase, Sirt1, is involved in cellular survival pathways, which ensure that the tumor suppressor gene, p53, and members of the forkhead transcription factor family remain deacetylated (15). Therefore, Sirt1 is considered to promote cancer genes and it may be involved in the regulation of tumor formation and initiation and developmental processes (15).

In order to further explore the significance and mechanism of Sirt1 in the pathogenesis of colorectal carcinoma, the present study investigated the expression of Sirt1 in colorectal carcinoma tissues and normal colorectal mucosa by means of the immunohistochemistry streptavidin peroxidase (SP) method and western blot analysis, and analyzed the associations between the expression levels of Sirt1 and clinicopathological factors. The results may provide novel ideas and inspiration for the research of colorectal cancer etiology.

Materials and methods

Research subjects. The present study was approved by the Affiliated Xinhua Hospital of Dalian University (Dalian, China) ethics committee. Specimens were collected with informed consent, and the investigation did not affect the disease diagnosis or follow-up treatment of the patients. Specimens from 40 patients undergoing surgical resection for the treatment of colorectal cancer were collected between March 2010 and October 2012 at the Affiliated Xinhua Hospital of Dalian University. The colorectal cancer tissue specimens represented the experimental group, and the adjacent normal mucosa tissues (>5 cm from tumor lesions) represented the control group. The patients included in the study had not previously received surgery for colorectal cancer, and did not exhibit endocrine or immune system disease. Patients who had received chemotherapy were excluded from the study. Hormonal treatment was also not conducted within three months prior to the surgery. Samples were acquired from the specimens within 30 min of surgical removal, and each sample was divided into two. One part of the sample was fixed in 10% formalin for the preparation of the paraffin specimens, and the expression of Sirt1 was examined in these samples by the immunohistochemical SP method. The second part of the sample was stored in liquid nitrogen, and the expression of Sirt1 protein in the fresh tissues was detected by western blot analysis. The clinical and pathological data characteristics of the patients were assessed, and the association between the expression of Sirt1 and the clinical pathological data was analyzed. The experimental group and the control group included a total of 80 samples from 40 patients, comprising 27 males and 13 females with a mean age of 59.37±10.05 years. Hematoxylin and eosin staining identified 12 cases of colon cancer and 28 cases of rectal cancer in the experimental group, whilst the control group samples were composed of normal mucosa. The 40 cases of colorectal cancer consisted of 22 cases of predominantly ulcerative type and 18 cases

of exophytic type tumors, with no cases of polypoid type tumors (16). According to histological grading (17), there were 15 cases of high differentiation, 19 cases of moderate differentiation and 6 cases of low differentiation. With regard to lymph node metastasis, there were 19 cases without lymph node metastasis and 21 cases with lymph node metastasis. According to Duke's classification (18), there were 18 cases of stage A+B and 22 cases of stage C+D.

Main reagents. The Sirt1 rabbit anti-human polyclonal antibody (cat. no. sc-15404) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The universal immunohistochemical Streptavidin-Peroxidase staining kit (cat. no. SP-9000), diaminobenzidine (DAB) developing liquid and mouse anti-human GAPDH monoclonal antibody (cat. no. TA-08) were purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd. (Beijing, China). RIPA strong lysis buffer, the protease inhibitor phenylmethanesulfonyl fluoride (PMSF), a BCA Protein Assay kit and a Beyo ECL Plus kit were purchased from Beyotime Institute of Biotechnology (Nanjing, China).

Immunohistochemical staining of paraffin sections using the SP method. Paraffin embedding and sectioning of colorectal cancer and normal mucosa were conducted. Goat serum blocking solution (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.) was applied dropwise at room temperature for 15 min. The Sirt1 primary antibody (1:100) was added and incubated with samples at 4˚C overnight. A horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (cat. no. ZDR-5307; 1:300; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.) was added to the samples and incubated at room temperature for 30 min. Each 4-μm section was treated with 50 μl horseradish peroxidase-labeled streptavidin working fluid (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.). After the DAB liquid had developed for 3 min, the nuclei were counterstained with hematoxylin (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.) for 40 sec, followed by dehydration. The dehydrated sections were cover-slipped with neutral gum for microscopic examination (Axiolab; Carl Zeiss AG, Oberkochen, Germany). The positive staining graph included with the kit served as the positive control, and phosphate-buffered saline replaced Sirt1 and served as the negative control.

Photographs were captured from 5 arbitrary fields in each section using a digital camera (Nikon COOLPIX S9500; Nikon Corporation, Tokyo, Japan), and the double-blind method was used for the data statistics. The percentage of positively stained cells from the total cells in each field was scored as follows: <1%, 0 points; 1-20%, 1 point; 21-50%, 2 points; and >50%, 3 points. The positive staining intensity was then scored as follows: No coloring, 0 points; pale yellow, 1 point; brown-yellow, 2 points; and sepia, 3 points. The product of these two scores served as the overall section staining score.

Western blot analysis. Tissue proteins were extracted using the RIPA strong lysis buffer and PMSF. The concentration of protein was measured according to the instructions of the BCA kit. The protein samples were analyzed by 10% SDS-PAGE. After transferring the samples to a polyvinylidene fluoride

Table I. Sirt1 staining score as detected by the immunohisto-chemical streptavidin peroxidase method.

Group	Cases	Sirt1 staining score
Normal tissues	40	1.15±0.419
Cancer tissues	40	2.62±0.537[a]

Staining score data are presented as the mean ± standard deviation. [a]P<0.05 vs. normal tissues. Sirt1, Silent information regulator 1.

Table III. Gray value of the Sirt1 protein as detected by western blot analysis.

Group	Cases	Sirt1/GAPDH gray value
Normal tissues	40	0.72±0.327
Tumor tissues	40	1.21±0.245[a]

Sirt1/GAPDH gray value data are presented as the mean ± standard deviation. [a]P<0.05 vs. normal tissues. Sirt1, Silent information regulator 1.

Table II. Association between Sirt1 expression and clinico-pathological data of patients with colorectal cancer.

Variable	Cases	Sirt1 staining score	P-value
Gender			0.084
Male	27	2.35±0.347	
Female	13	2.89±0.561	
Age, years			<0.0001
<50	16	1.64±0.401	
≥50	24	2.91±0.514	
Morphological type			<0.0001
Ulcerative	22	1.58±0.462	
Exophytic	18	2.93±0.618	
Tissue differentiation			<0.0001
High	15	1.46±0.471	
Medium or low	25	2.79±0.630	
Depth of invasion			<0.0001
Shallow muscle layer	19	1.60±0.513	
Whole layer	21	2.97±0.437	
Lymph node metastasis			<0.0001
Yes	21	1.53±0.428	
No	19	2.95±0.643	
Duke's stage			<0.0001
A+B	19	1.49±0.501	
C+D	21	2.92±0.713	

Sirt1 staining score data are presented as the mean ± standard deviation. Sirt1, Silent information regulator 1.

membrane (Beyotime Institute of Biotechnology), 5% skim milk powder was applied for blocking and samples were incubated at 4˚C overnight. After washing the membrane with Tris-Buffered saline with Tween 20, the protein samples were incubated with the polyclonal rabbit anti-human polyclonal Sirt1 (cat. no. sc-15404; Santa Cruz Biotechnology, Inc.) and monoclonal mouse anti-human GAPDH antibodies (cat. no. TA-08; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.) at 4˚C overnight, followed by the horseradish perox-idase-conjugated goat anti-mouse IgG secondary antibody (cat. no. ZDR-5307; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.) at 4˚C overnight. Subsequently, the membranes were placed in a chemiluminescence imaging instrument (ImageQuant LAS 4000 Mini; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA), then exposure and image capture and analyses were conducted (ImageQuant TL 1.0 software; GE Healthcare Bio-Sciences).

Statistical analysis. SPSS software version 22.0 (IBM SPSS, Armonk, NY, USA) was used for statistical analysis. The data are expressed as the mean ± standard deviation. A paired-samples t-test was employed to analyze the expression of Sirt1 in the colorectal cancer tissues and the normal mucosa tissues. One-way analysis of variance was performed to analyze the differences in Sirt1 expression in the tissues associated with the various clinical and pathological variables. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression levels of Sirt1 in paraffin-embedded colorectal cancer and normal mucosa samples detected by immunohis-tochemistry. As indicated by the arrows in Fig. 1, Sirt1 was primarily expressed in the nucleus, with stained nuclei indi-cating positive staining. As illustrated in Fig. 1B, Sirt1 was highly expressed in the colorectal cancer samples; the area of positive staining was large. By contrast, the expression of Sirt1 was weaker and the area of positive staining was more limited in normal mucosa samples (Fig. 1A).

The expression levels of Sirt1 in cancer tissues and normal mucosa were analyzed according to the evaluation standards. Multiplication of the staining percentage and staining intensity scores were conducted to calculate the mean overall section staining values of the samples in the experimental and control groups. A significant difference was identified between the two groups (P<0.05; Table I).

The associations between Sirt1 expression and clinico-pathological variables are presented in Table II. The mean staining score for Sirt1 was 2.35±0.347 (±standard deviation) and 2.89±0.561 in male and female patients, respectively. However, no significant differences in Sirt1 expression were identified between males and females (P=0.084). The mean staining score for Sirt1 was 1.64±0.401 and 2.91±0.514 in patients aged <50 and ≥50 years, respectively, and this differ-ence was determined to be statistically significant (P<0.0001). The mean staining score for Sirt1 in 22 patients with ulcerative type colorectal cancer was 1.58±0.462, while in 18 patients
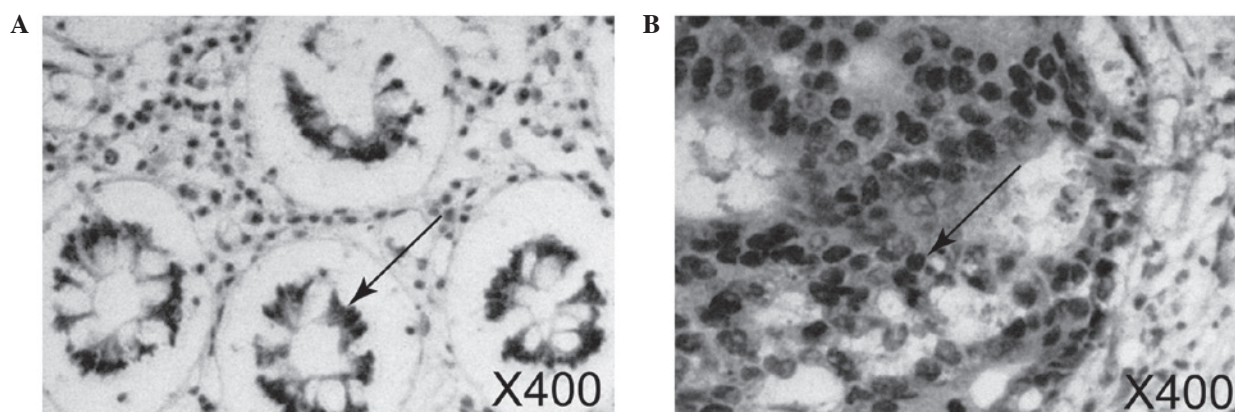
Figure 1. Immunohistochemical staining of Sirt1 in (A) normal mucosa tissue and (B) colorectal cancer tissue. Arrows indicate positive Sirt1 staining. Sirt1, Silent information regulator 1.
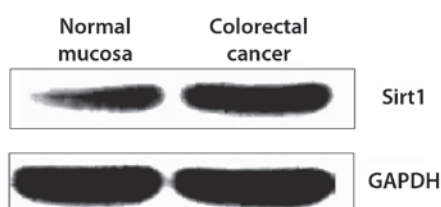

Figure 2. Expression of Sirt1 protein in colorectal carcinoma and normal mucosa tissues assessed by western blot. Sirt1, Silent information regulator 1.

with exophytic type the mean staining score for Sirt1 was 2.93±0.618. This difference was statistically significant (P<0.0001). The mean staining score for Sirt1 in 15 patients with highly differentiated cancer was 1.46±0.471, whereas in the 25 patients exhibiting medium and low differentiation, the mean staining score was 2.79±0.630, and this difference was found to be statistically significant (P<0.0001). Regarding tissue differentiation, the 19 patients with superficial muscular layer differentiation exhibited a mean Sirt1 staining score of 1.60±0.513, whereas the 21 patients exhibiting whole layer differentiation exhibited a mean score of 2.97±0.437, and this difference was determined to be statistically significant (P<0.0001). The mean staining score for Sirt1 in 21 patients with lymph node metastasis was 1.53±0.428, and in 19 patients with lymph node metastasis the score was 2.95±0.643. This difference was also determined to be statistically significant (P<0.0001). The mean Sirt1 staining score for the 19 patients with A+B Duke's stage cancer was 1.49±0.501, whereas the mean Sirt1 staining score of the 21 patients with C+D Duke stage cancer was 2.92±0.713, and this difference was statistically significant (P<0.0001). Therefore, the present results revealed that older age, lower tissue differentiation, deeper depth of invasion, lymph node metastasis and higher Duke's stage are associated with higher Sirt1 expression (P<0.0001). Furthermore, in terms of morphological type, Sirt1 expression is higher in exophytic compared with ulcerating tumors (P<0.0001); however, Sirt1 expression is not significantly associated with patient gender (P=0.084).

Expression of Sirt1 protein in fresh colorectal cancer and normal mucosa tissue samples detected by western blotting.

According to the detection results acquired by the SP immunohistochemistry method, Sirt1 may be an important factor in colorectal cancer, as indicated by the significantly increased expression of Sirt1 in the paraffin-embedded carcinoma specimens. To verify this observation, the expression of Sirt1 protein in fresh tumor tissues and normal tissues was analyzed simultaneously by western blot analysis. Expression was analyzed in 40 tissue specimens in each group, and the expression levels of Sirt1 were presented as the gray value of Sirt1/GAPDH. The results demonstrated that Sirt1 expression in colorectal cancer tissue was elevated compared with that of normal mucosa (Fig. 2; Table III). This difference was significant (P<0.05).

Discussion

The occurrence and pathogenesis of colorectal cancer constitute a complex process regulated by numerous genes (1). Under the conditions of different stimuli, abnormal transcription and translation of multiple genes induces changes in the expression of signaling proteins. Subsequently, signal conduction pathways become uncontrolled, leading to an imbalance in cell growth, survival, differentiation and proliferation (19). The protein acetylation/deacetylation cycle is also important in the process of gene expression and regulation (20). Protein acetylation and deacetylation are catalyzed by histone acetyltransferases and histone deacetylases, and Sirt1 belongs to the class III histone deacetylases and is a member of the Sirtuin family. This family catalyzes the deacetylation of lysine residues on various proteins in a NAD^+-dependent manner (10,21).

Multiple studies have demonstrated that the possible regulatory mechanism of Sirt1 as a cancer gene is associated with tumor protein p53 (22). The p53 protein is a tumor suppressor protein that serves diverse roles in multiple physiological processes within the body. Following a decrease in its expression levels, or if gene mutation occurs, the p53 protein can no longer fulfill these roles, thus increasing the risk of cancer (23). Sirt1 may induce p53 loss-of-function through the deacetylation of p53 at the Lys382 residue, which is contained in the C-terminus, consequently losing the ability to suppress tumor formation (24). Furthermore, studies have also demonstrated that, under conditions of DNA damage and oxidative stress, the

overexpression of Sirt1 may inhibit the p53-regulated cell cycle and lead to the arrest of cell replication and cell apoptosis (25). In addition, the deacetylase activity of Sirt1 was observed to decrease when Sirt1 mutants were transcribed and translated using the site-directed mutagenesis method in a model of carcinogenesis, which increased the sensitivity of cells to DNA damage and the oxidative stress response (26). Further studies demonstrated that the addition of a specific inhibitor of Sirt1, 5' adenosine monophosphate-activated protein kinase, into human hepatoma HepG2 and PLC/PRF/5 cells led to significant reductions in the activity and function of Sirt1, resulting in increased acetylation and transcriptional activity of the p53 protein (27,28). In addition, previous studies noted that Sirt1 expression in primary colorectal carcinoma was significantly increased, suggesting that Sirt1 is key in the occurrence and development of intestinal tumors (29). In the present study it was observed that Sirt1 expression in the primary colorectal cancer tissues were all significantly increased.

There are notable effects of living habits, diet structure and geographical regions on the incidence of gastrointestinal tract cancer (7,30). Therefore, in order to minimize the effect of external factors on the experimental results, the current study used colorectal cancer specimens and normal mucosa samples from the same patients as the experimental and control groups, respectively. The results demonstrated that, compared with that of normal mucosa tissues, the expression of Sirt1 in colorectal cancer tissues was significantly increased. Although gender did not affect the expression of Sirt1, significant differences in the expression of Sirt1 in colorectal specimens were observed between patients of different age groups, with significantly increased expression in patients aged ≥50 years. This may be associated with the putative role of Sirt1 as the longevity gene (31). Studies have indicated that the specific activators of Sirt1 contribute to the protection of cardiovascular function and prolong life (32). Therefore, the greater the age of the patient, the higher the expression of Sirt1 may be. The increased expression of Sirt1 was more evident in the process of cancer cells. Due to the fact that patients with polypoid type and adhesive type tumors were fewer in number, along with the greater difficulty of accessing the normal mucosa in such patients, the present research was conducted using tumors of ulcerative and exophytic gross morphological types. In addition, as the ulcerative type tumors with low differentiation accounted for a large proportion of the sample, the degree of malignancy was high. By contrast, the proportions of high and low differentiation in patients with the exophytic type tumor were comparable, and the degree of malignancy was low. It was observed that the expression of Sirt1 increased significantly in the patients with the ulcerative type. This is consistent with the observations for degree of tissue differentiation; increased Sirt1 expression was enhanced in the group of patients with medium and low differentiation. The depth of invasion, lymph node metastasis and Duke's stage are also indices reflecting the degree of cancer progression and malignancy (33). According to these three indices, it was observed that the expression of Sirt1 was elevated more significantly in cases with a higher degree of malignancy and further progression of colorectal carcinoma. Therefore, these results provide strong evidence of Sirt1 as a cancer-associated gene in colorectal cancer, laying the foundation for follow-up research.

However, opposing opinions have also been presented. One study suggested that the Sirt1 protein may serve inhibitory roles in the occurrence and development processes of colon cancer (34). In addition, animal tumor models have shown that Sirt1 may function as an anti-oncogene, acting as a tumor-inhibitory factor (35). Leko et al overexpressed the Sirt1 protein in APC$^{Min/+}$ mice, and the risk of cancer of the colon was significantly reduced (36). The mechanism underlying this may be that the overexpressed Sirt1 acts as a deacetylase, leading to the deacetylation of β-catenin in the cytoplasms of cells, thereby resulting in its nuclear localization and loss of its normal function (37). Furthermore, the expression level of Sirt1 protein in the nucleus and its deacetylation activity have been demonstrated to be negatively correlated with the expression of β-catenin (38).

In conclusion, the association between Sirt1 and the occurrence and development of cancer, and the related mechanisms, are unclear at present. The leading theory is that the activation of the Sirt1 protein can increase the risk of cancer, based on the evidence that Sirt1 can deacetylate and inactivate the tumor suppressor gene p53 (26). The present investigation provides a novel direction for investigation into the early diagnosis and targeted treatment of colorectal cancer.

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