Single Investigation of the Role of APC2 in Colorectal Cancer

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Research

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Abstract

Background

APC2 is as a homolog of adenomatous polyposis coli (APC) that has comparable functions in cancer, and is located on chromosome 19p13.3. It is an important signaling pathway protein in many cancers and diseases. For example, in ovarian cancer, APC2 serves as an important tumor suppressor through the induction of the WNT signaling pathway, inhibiting tumor invasion and growth. In colorectal cancer, APC2 was shown to be an important protein for inhibiting tumor invasion and metastasis. In the present study, APC2 was shown to be an important regulatory pathway protein that cooperates with microRNA bidirectional regulation to induce phenotypic changes in colorectal cancer.

Methods

Through measurements in colorectal cancer tissue samples (RT-PCR and immunohistochemistry), we found that the APC2 gene may play a role in the occurrence and development of colorectal cancer patients. We selected colorectal cancer cell lines as the research carrier of the APC2 gene. We changed the expression of APC2 gene in colorectal cancer cell lines by silencing the APC2 gene and up-regulating the APC2 gene, and then used cell cycle, MTT and western blot methods to measure its possible effects on colorectal cancer mechanisms.

Result

We found that the expression of APC2 in normal mucosal tissues in colorectal cancer tissues is was than that of matched colorectal cancer tissues, both at the protein and mRNA levels. We then tested colorectal cancer cell lines for gene silencing and up-regulation. We found that silencing the expression of the APC2 gene effectively advanced the cycle in colorectal cancer cells. In subsequent protein testing, we found that the proteins of the relevant cycle checkpoints changed accordingly. We found the opposite in cell lines after up-regulating the expression of the APC2 gene. By contrast, the SW480 cell line with k-ras mutation in the key pathway did not produce relevant changes by up-regulating the expression of the APC2 gene.

Conclusion

Low expression levels of the APC2 gene in colorectal cancer inhibit the progression of colorectal cancer cell lines through the RAS signaling pathway and hinders the occurrence and development of colorectal cancer.

Introduction

Research on colorectal cancer has focused on the treatment of patients with high-stage colorectal cancer in the late stages of surgery from the appearance of staged surgical methods. This can inhibit the growth
of tumors; however, not all cancer patients can tolerate surgery, and targeted drugs for cancer development have become the focus of research.

APC2 is a homolog of adenomatous polyposis coli (APC) that has comparable functions in cancer; it is located on chromosome 19p13.3 (1). It is an important signaling pathway protein that has been reported in many cancers and diseases. For example, in the study of ovarian cancer, APC2 serves as an important tumor suppressor through the induction of the WNT signaling pathway to inhibit the biological behavior of tumor invasion and growth (2). In colorectal cancer, APC2 is an important protein for inhibiting tumor invasion and metastasis (3). It is an important regulatory pathway protein that it cooperates with microRNA bidirectional regulation to induce phenotypic changes in colorectal cancer.

Many studies have used APC2 as a gene similar to APC, looking for associations and similarities between its analogs in the protein pathway, ignoring that it may also play an important role as a protein. In the present study, we focused on the possible role of APC2 protein and its independent association with colorectal cancer.

**Method And Materials**

**Patients and cell lines**

The research cohort recruited at Xi’an First Hospital from September 2018 to mid-September 2019. These were colorectal cancer patients who were treated not received radiotherapy and chemotherapy before the removal of tissue samples. Before the operation, the patients signed and agreed to participate in the study by signing informed consent forms distributed by the ethics committee of this hospital. After tissue samples were removed, some portions were stored in liquid nitrogen, and some were embedded in wax blocks for later use. The colorectal cancer cell lines and colorectal normal mucosal tissue cell lines selected in this experimental group were purchased from Shanghai Cell Bank.

**Transfection**

APC2 interfering RNA was purchased from Shanghai Shenggong. We used control and interfering RNA to silence the APC2 gene in the HT29 cell line, and then used it for protein and cell cycle experiments. Subsequently we referred to the lines as Sil-control and Sil-APC2. APC2 plasmids and its control group were purchased from Origen, and we later used them to up-regulate APC2 gene expression in HCT8 and SW480 cell lines. PCMV6 and PC-APC2 were used to refer to the up-regulated HCT8 cell lines. PCMV6-SW480 and PC-APC2-SW480 refer to the up-regulated SW480 cell lines. The transfection reagent lipo3000 was purchased from Thermo Scientific.

**Real Time PCR**
TRIzol reagent (Takara Bio, Shiga, Japan) was used to isolate total RNA from frozen cancer tissues. To do this, we added 1 ml of TRIzol reagent and 300 µl of chloroform to the tissue, mixed, and then centrifuged at 10,000 rpm for 15 minutes. Next, an equal volume of isopropanol was added to the supernatant, and then the sample was centrifuged again at 10,000 rpm for 15 minutes. The supernatant was discarded and the precipitate was washed with 1 ml of 75% ethanol. Next, we followed the manufacturer's instructions (Takara) to reverse transcribe the extracted RNA using the kit. Then, we designed the amplification primers for the messenger APC2 based on the specific sequence (Shanghai, China). The expression of mature APC2 was measured using the thermal cycler Dice real-time system II under the following thermal cycling conditions: 95 °C for 30 s, 45 cycles, 95 °C for 5 s, 60 °C for 60 s, and then conducting melting curve analysis. The relative expression of APC2 was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase mRNA, and the fold-change in expression was calculated using the $2^{\Delta\Delta Ct}$ method. Compared with adjacent non-tumor tissues, we defined negative values as relatively low expression levels of APC2, and positive values as relatively high expression levels of CRC of APC2.

**Immunohistochemistry**

From the resected tumors of patients with colorectal cancer, thin paraffin sections were prepared, baked in a 70-degree oven for 2 hours, and then dewaxed (xylene-pure ethanol-75% ethanol for 30 minutes each). According to the instructions of Maixin reagent immunohistochemistry, we added solution A (catalase) dropwise to eliminate non-atopic staining (30 minutes), rinsed with PBS three times and incubated solution B (Ultra V Block) for about 30 minutes, then added primary antibody, and stored at 4 °C overnight. On the second day, we rinsed with PBS solution 3 times, incubated with solution C (Primary Antibody Enhancer) for about 15 minutes, rinsed with PBS solution 3 times, and incubated with solution D (enzyme labeled secondary antibody) for about 15 minutes. DAB chromogenic solution was stained for 5 minutes, then concentrated hydrochloric acid was added for 6 seconds, hematoxylin was stained for 5 minutes, and alkaline water turned blue for 3 minutes. After the final dehydration (75% ethanol-pure ethanol-xylene for 30 minutes each), the seals are retained.

Scoring: We observed the stained sections under a microscope. After selecting the field of view, according to the staining intensity, very strong = 3 points, strong = 2 points, weak = 1 point, and none = 0 points. Evaluation of the range of dyeing: 100–75%: 4 points, 74–50%: 3 points, 49–25%: 2 points, 24–0%: 1 point. Those with a total score equal to or greater than 6 points were judged to have strong expression.

**Cell cycle**

After 48 hours, the cells transfected with iRNA or plasmids were trypsinized into centrifuge tubes, rinsed 3 times with PBS solution, and transferred to the cell cycle staining solution (PI), protected from light at 37 degrees for 30 minutes, and then placed in a bleed cytometer for detection, and the results obtained are
analyzed and detected by software. Cell cycle reagent stains were purchased from Thermo Fisher Scientific.

**MTT**

In the MTT experiment, after 24 hours of transfection, the cell line was eluted, and it was seeded into 96-well plates at 2500 cells per well, cultured for 5 days, and medium was changed daily (5 plates repeat one of the plates). After cleaning the medium, we added DMSO to the microplate reader for detection. The experiment was performed three times, and the data analysis is presented below.

**Western blot and co-immunoprecipitation**

After culturing cells for 48 hours in PBS, we washed them three times and incubated them in RIPA buffer for 30 minutes on ice. After centrifugation, we removed the supernatants and tested on a microplate reader to quantify with standard protein. To the same concentration for use, after configuring the electrophoresis gel, we dropped the sample into wells of the gel plate in the same volume (30 µl) one-by-one and started the electrophoresis process to the loading mark to the bottom of the gel plate, then cut the gel according to MARKER and transferred onto PVDF membranes. Membranes were then washed 3 times with TBST solution (Thermo Fisher Scientific), and primary antibody was incubated overnight at 4 degrees. After washing 3 times with TBST solution, the rabbit secondary antibody was incubated and incubated at room temperature for 2 hours. After washing thoroughly, we used luminescent solution (Thermo Fisher Scientific) in an ECL luminometer (Bio-Rad) for detection.

**Statistical analysis**

Patient tumor-related information and immunohistochemical scores were evaluated using $\chi^2$ tests, and expression levels of related mRNA and protein were measured using the independent sample T-test after obtaining quantitative data. with p values less than 0.05 were considered significant. All data analyses were performed using SPSS 17.0.

**Results**

**Protein expression levels of APC2 gene were higher in normal colonic mucosa tissue**

In this experimental group, the APC2 protein was used as the research focus, and colorectal cancer tissues retained in wax blocks were stained for immunohistochemistry (Fig. 1). Positive expression levels of mucosal tissue were higher, while that of cancerous tissue next to it expressed APC2 protein at lower levels. Subsequently, we also tested mRNA levels transcribed by the APC2 gene, and calculated the
relative expression (cancer tissue-normal mucosal tissue) data. We found that mRNA expression levels of cancer tissue were lower than those of normal tissue.

**APC2 gene expression in colon cancer-related cell lines**

Colorectal cancer cell lines (HCT-116, SW480, HT29, HCT8) and normal mucosal tissue cells (HIEC) purchased from the Shanghai Cell Bank were used for our measurement of APC2 gene expression. Western blot was first used to measure rubber plate protein expression levels, then we used RT-PCR to measure mRNA expression levels of APC2 in cell lines. We found that the expression levels of the APC2 gene protein in colorectal cancer cell lines were lower than those in HIEC, at the protein and mRNA levels. Protein levels of APC2 gene were higher in HT29, and levels in HCT8 were lower. (Figure 2) Therefore, we chose these two cell lines as research vectors to regulate the APC2 gene.

**APC2 protein expression positively correlated with P53 expression**

According to the genetic test results of the pathology department on patient histology, we conducted genetic testing of key proteins in the tissue samples of 100 patients with colorectal cancer. We selected growth-related genes (P53 and Ki67), and used the immune group. Tissue samples of colorectal cancer patients were tested for two genes (Fig. 3). After scoring data analysis, we found that the expression of APC2 protein was positively correlated with that of P53 protein and that of Ki67 (Table 1). During the analysis of the expression between the two, we found that the data had no meaning and the connection between APC2 protein and Ki67 protein could not be judged.

| Parameter | P value | APC2 |  |
|-----------|---------|------|---|
|           |         | low  | high |
| P53       | 0.007   | 19   | 4  |
|           |         | 37   | 40 |
| Ki67      | 0.006   | 30   | 36 |
|           |         | 26   | 8  |
APC2 protein expression is negatively correlated with T stage of colorectal cancer patients

After comprehensive analysis of the immunohistochemical results, we compared patient clinical information to the high and low groups of the immunohistochemical score. After comprehensive analysis using the chi-square test, we found that stronger expression APC2 was associated with lower T Staging (p = 0.019, Table 2), size (p = 0.004 Table 2), N, and M staging; other clinical information was not meaningful for data analysis.

Table 2

| Parameter               | P value | CNN2  |
|-------------------------|---------|-------|
|                         |         | low   | high |
| Age(year)               |         |       |      |
| < 65                    | 0.337   | 24    | 24   |
| ≥ 65                    |         | 32    | 20   |
| Gender                  |         |       |      |
| Male                    | 1.000   | 27    | 21   |
| female                  |         | 29    | 23   |
| T stage                 |         |       |      |
| 1                       | 0.019   | 5     | 6    |
| 2                       |         | 15    | 22   |
| 3                       |         | 27    | 15   |
| 4                       |         | 9     | 1    |
| N stage                 |         |       |      |
| 0                       | 0.905   | 18    | 16   |
| 1                       |         | 22    | 16   |
| 2                       |         | 16    | 12   |
| M stage                 |         |       |      |
| 0                       | 0.872   | 41    | 52   |
| 1                       |         | 46    | 61   |
| Differentiation         |         |       |      |
| poor                    | 0.491   | 12    | 14   |
| medium                  |         | 18    | 13   |
| well                    |         | 26    | 17   |
| Tumor size(cm)          |         |       |      |
| < 5                     | 0.004   | 17    | 27   |
| ≥ 5                     |         | 39    | 17   |
**APC2 silencing advances the progression of cell cycle of colorectal cancer cell line**

Because the HT29 cell line expresses a higher protein expression of the APC2 gene, we chose it as the siRNA to silence the expression of the APC2 gene protein. In addition, we chose the weakly expressed HCT8 cell line as the carrier for the up-regulation of APC2 by plasmids. Because we found that APC2 protein may affect the P53 signaling pathway, and the P53 signaling pathway involves K-ras, a key signaling pathway protein for intestinal cancer development, we also choose SW480G12V (KRAS G12V mutation) according to local conditions [4] as a cell line up-regulated by plasmids. We subjected the adjusted cell line to flow cytometry. We found that, in the cell line in which the APC2 gene was knocked out (HT29), cells in the G0-G1 phase advanced significantly, while the reverse effect was observed in the upregulated APC2 cell line (HCT8). Surprisingly, in the K-ras mutant cells, the cell cycle changes were not significantly different from those of the control group (Fig. 4).

**APC2 silencing advances the proliferation of colorectal cancer cell line**

After we interfered with the cell line, we tested its proliferation ability by counting cell colonies and using the MTT assay. We found that, in the cell colony experiment, the number of colonies in the cells that silenced APC2 increased significantly. However, the number in the up-regulated HCT8 cell line was significantly lower in the comparison control group. No significant changes were observed in the SW480 cell line mutated by K-ras mutant. In the MTT experiment we carried out for up to 5 days, APC2 silencing significantly increased the value-added ability of the cells, while the up-regulated cell lines showed significant proliferation fatigue. Similarly, no obvious changes were observed in the SW480 cell line mutated by K-ras mutant (Fig. 4).

**APC2 silencing inhibits P53 signaling pathway activation cycle-related protein**

After measuring interference, cell extract proteins were placed on gel plates for measurement. We found that, after APC2 silencing, P53 protein expression was inhibited, and the cycle-related pathway protein was activated, suggesting changes in the cell cycle and promotion of proliferation. However, after APC2 was up-regulated by plasmids, the P53 gene protein expression was up-regulated and cyclin expression was suppressed. Similarly, no clear differences were observed in the SW480 cell line (Fig. 5).

**Discussion**

Colorectal cancer (CRC) is the third most common cancer diagnosed in both males and females in the US (after prostate cancer in men, breast cancer in women and lung cancer). Approximately 41% of all...
colorectal cancers occur in the proximal colon, with approximately 22% involving distal colon and 28% involving rectum [5]. Although we can now use radical surgery for the treatment of early colorectal cancer, we lack effective treatments for colorectal cancer at higher stages [6]. Therefore, we focused on the study of the mechanisms of tumorigenesis and development, and provided new ideas for drug-targeted therapy of advanced tumors.

At present, there are many studies on tumors with respect to APC2. For example, our research in non-small cell lung cancer found that histone methyltransferase G9a inhibited the growth of non-small cell lung cancer and other biological phenomena by regulating the expression of APC2 [7]. In a study of lung cancer, miR-4326 promoted cell proliferation through targeting tumor suppressor APC2 [8]. Many studies found that APC2 is an analog of the APC gene and is an important key protein in the WNT signaling pathway to inhibit the occurrence and development of colorectal cancer. They did not use APC2 as the central protein studied separately; therefore, we chose to independently interfere with changes in the APC2 protein to measure changes in colorectal cancer cell lines.

In the present study there were two main findings, one "changed" and one "unchanged." We found that silencing of APC2 promoted cell cycle progression in colorectal cancer cell lines, and promoted the value-added ability of colorectal cell lines (Figure ). We were pleasantly surprised that the silencing of APC2 also significantly expressed the expression of cycle-related proteins P53 and cyclin D1. These increases suggest that APC2 may promote proliferation of colorectal cancer cell lines [9,10,11,12].

Using immunohistochemistry, we selected two common genes (P53 and Ki67). Using the chi-square test method, we found a positive relationship between the P53 gene expression and that of the target gene (APC2). In previous studies, there was a close connection between the P53 signaling pathway and the K-ras protein signaling pathway [13.14.15]. This pathway is particularly important in colorectal cancer cell lines [16].

Therefore, after extracting the proteins that interfere with the cell line, we not only measured expression levels of cycle-related proteins, but we also measured changes of two key pathway proteins, P53 and K-ras. We were surprised to find that silencing APC2 activated the K-ras signaling pathway, causing changes in downstream proteins and promoting the cycle. [17.18.19.20]. However, in the SW480 cell line of K-ras mutant that we specially selected for K-ras gene research, there was no interference with APC2 to cause a clear change in the cell line.

The K-ras mutant of sw480 cells has been reported in the literature [4]. In the literature, it is reported that the wild-type RAS gene changed the drug resistance and cell viability of colorectal cancer cell lines by loading the exogenously mutated RAS gene sequence; in the present study, we reported we focused on cell cycle detection, and we found that, in the SW480 cell line, there were no changes in cycle-related proteins after changing the expression levels of APC2 protein, as measured by flow cytometry. Whether the interference caused by this mutation APC2 alters the expression of K-ras gene has not yet been confirmed in the experiment, and further research is needed.
Declarations

Ethics approval and consent to participate

The Ethics Committee of the Xi’an First Hospital approved the research project and analysis of the patient specimens for this study.

Availability of data and materials

Please contact the corresponding author for data requests.

Funding

Not applicable

Competing interests

The authors have no competing interests.

Informed consent

All patients have signed an informed surgical consent before surgery. Informed consent includes the possibility of using patient specimens for clinical trial research, and promises to keep patient information and privacy completely confidential.

Author Contribution

The researchers have a clear division of labor and outstanding contributions. As co-first authors, Yingwei Jiao and Qiang Liu participated in the experiment design and thought guidance, and Hongbo Zhao Xianzhen Hu and Jinlong Sun participated in the experiment implementation and data collection. As corresponding authors, Yujie Fang participated in the overall arrangement and funding of the entire experimental idea.

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Figures

Figure 1

Protein expression levels of APC2 gene were higher in normal colonic mucosa tissue A. After staining the colorectal cancer tissue and the corresponding normal mucosal tissue by immunohistochemical method, we found that the colorectal cancer tissue expressed lower APC2. B, Real-time PCR is used to detect APC2 mRNA expression in colorectal cancer tissues and corresponding normal tissue mucosa. Similarly, colorectal cancer tissues express lower levels of APC2 mRNA.

Figure 2
APC2 gene expression in colon cancer-related cell lines A, the expression level of APC2 protein in 5 colorectal cell lines. B, the data expression form of APC2 protein through the calculation of gray value. C, APC2 mRNA expression levels in 5 colorectal cell lines.

Figure 3

APC2 protein expression positively correlated with P53 expression The expression of p53, Ki67 and APC2 in the same colorectal cancer tissue was detected by immunohistochemistry.
Figure 4

APC2 silencing advances the proliferation of colorectal cancer cell line A. Silencing the APC2 gene in HT29 cells and transfecting the SW480 and HCT8 cell lines with APC2 plasmids, then place them on a flow cytometer to detect the cell cycle. B, Data analysis of the G0-G1 phase of the cell cycle. C, MTT experimental data analysis of the above-mentioned transfected cells.
**Figure 5**

APC2 silencing inhibits P53 signaling pathway activation cycle-related protein A, Detect pathway-related proteins in the transfected cell line by western blot. B, the gray value analysis of each protein in the left picture.