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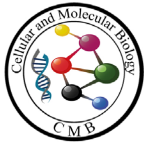

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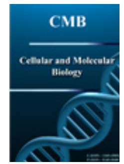

Original Article

In vivo and in vitro studies on the role of regulating Smac expression in the occurrence and development of colon cancer

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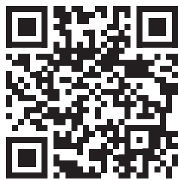

Abstract

We aimed to explore the role of regulating Smac expression levels in the occurrence and development of colon cancer through in vitro and in vivo experiments. Colon cancer cells HT-29 were cultured and transfected into different groups. qRT-PCR was used to detect the expression level of Smac in cells; Flow cytometry was used to detect the apoptotic ability of each group of cells; Western blot was used to detect the protein expression of Smac and apoptosis-related factors Survivin and Caspase-3; The nude mouse tumorigenesis experiment was conducted to detect the regulatory effect of regulating Smac expression levels on the growth of colon cancer transplanted tumors in vivo. In comparison to the FHC group, the HT-29 group exhibited a decrease in Smac expression. The si-Smac group, when compared with the si-NC group, showed significant reductions in Smac mRNA and protein levels, weaker cell apoptosis, increased Survivin, and decreased Caspase-3 expression. Contrarily, the oe-Smac group, against the oe-NC group, displayed increased Smac mRNA and protein levels, enhanced apoptosis, reduced Survivin, and elevated Caspase-3 expression. In nude mice tumor transplantation experiments, the LV-sh-Smac group, as opposed to the LV-sh-NC group, had tumors with greater volume and weight, reduced Smac and Caspase-3, and increased Survivin expression. In contrast, the LV-oe-Smac group, compared with the LV-oe-NC group, showed tumors with decreased volume and mass, increased expressions of Smac and Caspase-3, and decreased Survivin. Smac is lowly expressed in colon cancer. Upregulation of Smac expression can inhibit the occurrence and development of colon cancer, possibly by inhibiting Survivin expression and promoting Caspase-3 expression, thereby enhancing the pro-apoptotic function.

Keywords: Smac; Colon cancer; Occurrence and development; Cell apoptosis; In vivo and in vitro experiments

1. Introduction

Colon cancer, as one of the most prevalent types of malignant tumors of the digestive system in the world, has become a common cause of cancer death worldwide. Due to its high morbidity and mortality, colon cancer poses a major global public health challenge. This cancer usually originates in the lining of the colon or rectum and may develop into more severe forms over time. The survival rate of colon cancer is often affected due to its advanced diagnosis and complex treatment process. Therefore, early detection and effective treatment strategies are essential to reduce the mortality of colon cancer. As medical research continues to advance, so does the understanding and treatment of the disease, but it remains a major focus of global health concerns [1].

In clinical practice, widespread implementation of screening colonoscopy is an effective measure that helps to reduce the incidence of colon cancer. This screening procedure allows early detection of colon cancer or its precursor lesions, such as polyps, thus providing timely opportunities for intervention. In addition, colonoscopy screening also helps in the early diagnosis of cancers that have already formed, and treatment outcomes are generally better and patient survival is better when the cancer is in an early stage. Therefore, as a preventive strategy, regular screening colonoscopy plays an important role in controlling the incidence of colon cancer [2]. At the same time, various clinical treatment strategies, including resection, chemotherapy and immunotherapy, can help to improve the survival rate of patients [3]. However, considering the occurrence of colon cancer is one of the many factors involved in the pathological process of [4], and its diagnosis and treatment is still not satisfactory, the prognosis is not optimistic [5], it is necessary for the development of colon cancer underlying mechanisms for further in-depth.

Previous studies have confirmed that the occurrence and development of tumors are closely related to the biological characteristics of tumor cells [6,7]. The proliferation, invasion and apoptosis of tumor cells are directly related to the occurrence and progression of tumors [8]. As an im-

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portant regulator of apoptosis derived from mitochondria, Smac has been found to be the only pro-apoptotic protein widely present in mammalian cells [9]. Its pro-apoptotic effect is usually dependent on its ability to relieve the inhibitory effect of IAPs on the apoptotic executive protein Caspase-3 [10]. Therefore, the aim of this study is to explore the role of Smac in the occurrence and development of colon cancer in vitro and in vivo, in order to provide data reference for the mining of the molecular mechanism of colon cancer and the screening of key molecular targets.

2. Materials and Methods

2.1. Study design

Normal human colon epithelial cell line FHC and colon cancer cell line HT-29 were taken and cultured normally. The expression levels of Smac in FHC and HT-29 cells were confirmed by qRT-PCR. Colon cancer HT-29 cells in good growth condition were further transfected into the following groups: si-NC group and si-Smac group; oe-NC group and oe-Smac group. Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.2. qRT-PCR

RNeasy Mini Kit was used to extract total RNA. The qRT-PCR reaction was performed using a real-time fluorescent quantitative PCR instrument. The PCR amplification program was a two-step method: predenaturation at 95 °Cfor 30 s; The PCR reaction, at 95 °C for 5 s, 60 °C, 34 s in 40 cycles in a row, repeat each sample set three holes. The Ct values of each well were recorded, and the relative expression of products was calculated by the 2-ΔΔCt method using GAPDH as an internal control. ΔΔCt = (experimental purpose gene Ct average - experimental housekeeper Ct average) - (control, 0 • Ct average - gene control housekeeping gene Ct average).

2.3. Western blot

Total protein was extracted from RIPA lysate, and incubated on ice for 30min, and the supernatant was removed after centrifugation at 8000g for 10min. Total protein concentration was determined by BCA kit. SDS-PAGE gel electrophoresis was performed, and proteins were transferred to PVDF membranes by wet rotation and blocked with 5% skim milk powder for 1 h at room temperature. Then PVDF membrane and dilution of a rabbit single resisting Smac antibodies (ab32023, latter, abcam, Cambridge, UK), Survivin (ab134170, latter, abcam, Cambridge, UK) and Caspase-3 (ab32351, 1:500, abcam, Cambridge, UK) and the reference β-actin (ab6276, 1:500, abcam, Cambridge, UK) were incubated overnight at 4°C. After washing three times with TBST, the PVDF membrane was incubated for 1 h with HRP-labeled secondary anti-IgG antibody (ab133470, 1:2000, abcam, Cambridge, UK). ECL fluorescence detection kits, gel imaging exposure imaging. Relative protein content was expressed as gray value of Smac protein band/gray value of β-actin protein band.

2.4. Cell apoptosis

Cell apoptosis was detected by flow cytometry. The culture supernatant was collected and digestion was terminated after trypsin digestion. Centrifugation was performed at 1500 rmp speed for 5 min and repeated after PBD cleaning. The cell precipitate was washed with the addition of binding buffer and the centrifugation was repeated. After resuspension, the cell suspension was taken and subjected to Annexin V-APC staining and PI staining. They were transferred to a flow tube for detection.

2.5. Animals

Forty female B-Alb /C nude mice (18-20 g) aged 5 weeks were randomly divided into 4 groups: LV-sh-NC group and LV-sh-Smac group. LV-oe-NC group and LV-oe-Smac group, 10 rats in each group. Stable cell lines obtained by lentivirus infection were suspended in PBS and inoculated subcutaneously into the ventral side of nude mice. Tumor volume was calculated according to (a×b2) /2 (a is the longest diameter of tumor, b is the shortest diameter of tumor). Thirty days after inoculation, the nude mice were killed by carbon dioxide asphyxia and the tumors were excised and weighed. All experimental procedures were approved by the Animal Care Experimental Committee of our hospital, followed the ethical convention on experimental animals and conformed to the relevant national regulations.

2.6. Statistical analysis

All data were processed by Statistic Package for Social Science (SPSS) 21.0 software (IBM, Armonk, NY, USA), measurement data were expressed as mean ± standard deviation, and paired t-test was used. With significant difference ($P < 0.05$) said statistical significance.

3. Results

In order to detect the expression of Smac in colon cancer cells, FHC group and HT-29 group were set up in this experiment, and the expression of Smac was detected by qRT-PCR. The results showed that compared with FHC group, the expression of Smac in HT-29 group was decreased (1.00±0.05 vs. 0.43±0.02; $P<0.05$), suggesting that Smac is lowly expressed in colon cancer and may play a role in promoting cancer. Up-regulation of Smac expression can inhibit the apoptosis of colon cancer cells. In order to verify the Smac expression in its role in the development of colon cancer, we made the following experiment. We set up si-NC group and si-Smac group in HT-29 cells. oe-NC group and oe-Smac group. The mRNA and protein expression of Smac was detected by qRT-PCR and western blot (Tables 1 and 3). The results showed that compared with the si-NC group, the mRNA and protein expression of Smac in the si-Smac group decreased significantly (both $P<0.05$). Compared with the oe-NC group, the mRNA and protein expression of Smac in the oe-Smac group were significantly increased (both $P<0.05$).

At the same time, flow cytometry was used to detect the apoptotic ability of HT-29 cells in each group (Table 2). The results showed that compared with the si-NC group, the apoptotic ability of HT-29 cells in the si-Smac group was significantly reduced. However, compared with the oe-NC group, the apoptosis ability of HT-29 cells in the oe-Smac group was significantly enhanced. In addition, the protein expression of apoptosis-related factors Survivin and Caspase-3 was detected by western blot (Table 3). The results showed that compared with the si-NC group, the expression of Survivin protein in the si-Smac group was significantly increased, while the expression of Caspase-3 protein was significantly decreased (both $P<0.05$).

Compared with the oe-NC group, the expression of Survivin protein in the oe-Smac group was significantly decreased, while the expression of Caspase-3 protein was significantly increased (both P<0.05).

To verify the effect of up-regulation of Smac expression on colon cancer in vivo, we set up LV-sh-NC group and LV-sh-Smac group in BALB/c nude mice. In the LV-oe-NC group and LV-oe-Smac group, the cells with stable virus transformation were subcutaneously inoculated into nude mice, respectively. The nude mice were sacrificed to obtain the transplanted tumors, and the tumor volume and mass data were obtained. Statistical analysis showed that compared with the LV-sh-NC group, the LV-sh-Smac group had significantly increased tumor volume and weight. Compared with the LV-oe-NC group, the volume and weight of the LV-oe-Smac group were significantly reduced (all P<0.05; Table 4).

Finally, the expression of Smac in the transplanted tumor tissues of each group was detected by qRT-PCR assay and the protein expression of Smac, Survivin and Caspase-3 was detected by WB (Tables 5 and 6). The results showed that compared with the LV-sh-NC group, the expression of Smac and Caspase-3 in the LV-sh-Smac group decreased significantly, while the expression of Survivin increased significantly (all P<0.05). Compared with the LV-oe-NC group, the expression of Smac and Caspase-3 in the LV-oe-Smac group increased significantly, while the expression of Survivin decreased significantly (all P<0.05).

Table 1. qRT PCR detection cell transfection Smac mRNA expression in the model.

Group	mRNA expression
si-NC Group	1.00±0.06
si-Smac Group	0.50±0.04*
oe-NC Group	0.99±0.05
oe-Smac Group	1.77±0.09#

* indicates P<0.05 compared with si-NC group and # indicates P<0.05 compared with oe-NC group.

Table 2. The apoptotic capacity of the cells in each group was determined by flow cytometry.

Group	Apoptosis rate
si-NC Group	9.75±1.39
si-Smac Group	4.22±0.68*
oe-NC Group	10.48±1.52
oe-Smac Group	19.27±2.76#

* indicates P<0.05 compared with si-NC group and # indicates P<0.05 compared with oe-NC group.

Table 3. Western blot analysis of protein expression of Smac and apoptosis-related factors Survivin and Caspase-3 in the cell transfection model.

Group	Smac	Survivin	Caspase-3
si-NC Group	1.54±0.08	1.21±0.05	1.73±0.09
si-Smac Group	0.87±0.06*	1.99±0.12*	1.11±0.05*
oe-NC Group	1.60±0.08	1.17±0.06	1.76±0.08
oe-Smac Group	2.19±0.16#	0.65±0.04#	2.69±0.21#

* indicates P<0.05 compared with si-NC group and # indicates P<0.05 compared with oe-NC group.

Table 4. Transplanted tumor volume and mass data of nude mice in each group.

Group	The volume of transplanted tumor (mm^3)	Quality of transplanted tumor (g)
LV-sh-NC Group	843.28±43.25	1.14±0.05
LV-sh-Smac Group	1189.39±54.37*	1.43±0.07*
LV-oe-NC Group	850.12±44.78	1.09±0.06
LV-oe-Smac Group	505.20±29.46#	0.47±0.03#

* indicates P<0.05 compared with si-NC group and # indicates P<0.05 compared with oe-NC group.

Table 5. qRT-PCR detection Smac mRNA expression in the nude mouse transplantation tumor model.

Group	mRNA expression
LV-sh-NC Group	1.00±0.04
LV-sh-Smac Group	0.57±0.03
LV-oe-NC Group	0.97±0.05
LV-oe-Smac Group	1.69±0.09

* indicates P<0.05 compared with si-NC group and # indicates P<0.05 compared with oe-NC group.

Table 6. Western blot analysis of protein expression of Smac and apoptosis-related factors Survivin and Caspase-3 in the xenograft tumor model of nude mice.

Group	Smac	Survivin	Caspase-3
LV-sh-NC Group	1.68±0.07	1.24±0.06	1.82±0.09
LV-sh-Smac Group	1.04±0.05*	1.87±0.08*	1.32±0.05*
LV-oe-NC Group	1.71±0.06	1.19±0.07	1.85±0.10
LV-oe-Smac Group	2.43±0.18#	0.66±0.04#	2.76±0.25#

* indicates P<0.05 compared with si-NC group and # indicates P<0.05 compared with oe-NC group.

4. Discussion

Colon cancer is a common malignant tumor originating from the digestive tract. Genetic and epigenetic changes, adverse lifestyle, environmental factors, and inflammatory bowel disease may increase the risk of colon cancer [11]. Cell apoptosis is one of the physiological cell death processes, in the process of maintaining the stability of the environment within the body function key [12]. Cell apoptosis is restrained, which can lead to cell survival, and provide favorable conditions for transformation of mutated cells to accumulate, eventually leading to the occurrence of tumor [13]. Previous studies have found that Smac plays an important role in tumor cell growth by enhancing Caspase-3 activity and down-regulating Survivin expression through the interaction between IAP molecules [14,15].

In this study, normal human colonic epithelial cell line FHC and colon cancer cell HT-29 were used for in vitro cell experiments. And apply the qRT - PCR detection of Smac in cells, the expression level; Flow cytometry detection of each cell apoptosis ability; Western blot detection Smac and apoptosis factor of Survivin and Caspase 3 protein expression. It was found that Smac expression was decreased in colon cancer HT-29 cells compared with normal human colon epithelial cell line FHC, suggesting that Smac expression is low in colon cancer. Further transfection experiments showed that after inhibiting the expression of Smac, the mRNA and protein expressions of Smac were significantly decreased, the ability of apoptosis was significantly weakened, the protein expression of Survivin was significantly increased, and the protein expression of Caspase-3 was significantly decreased. After the expression of Smac, considerably increased the Smac mRNA and protein expression, markedly enhancing its capability of cell apoptosis, Survivin protein expression decreased significantly, and considerably increased the Caspase 3 protein expression. Into at the same time, this study adopts the nude mice tumor experiment testing control Smac expression levels in the body and the regulation of the colon cancer transplantation tumor growth. The results showed that after inhibiting the expression of Smac in nude mice, the transplanted tumor volume and weight were significantly increased, the expression of Smac and Caspase-3 was significantly decreased, and the expression of Survivin was significantly increased. After promoting Smac expression, the tumor volume and weight of nude mice were significantly reduced, the expression of Smac and Caspase-3 was significantly increased, and the expression of Survivin was significantly decreased. Based on the above studies, we speculate that the down-regulation of Smac expression, the increase of Survivin overexpression, and the decrease of Caspase-3 expression may enhance the ability of the body to inhibit apoptosis and decrease the pro-apoptotic function, breaking the balance between cell proliferation and apoptosis, and then promote the occurrence of colon cancer. While raising Smac expression, increase Caspase 3 expression, Survivin, mentioning enhancements, promoting apoptosis reduced cell survival, thereby inhibiting tumor growth. As a common apoptosis-related factor, the role of Smac in the occurrence and development of tumors has been extensively studied [16,17]. In terms of its molecular mechanism in tumor pathogenesis, it has been found that cIAP inhibits the activation of exogenous apoptotic pathways by preventing the formation of pro-apoptotic signaling complexes, while Smac can induce the degradation of cIAP [18]. The combination of non-genotoxic SmAC-mimics and glycolysis inhibitors can play an important role in IAP-mediated cancer cell survival [19]. Scholars for cancer chemotherapy drug resistance of common issues in treatment and failure in which the role of tumor cell apoptosis, through the study found that IAPs and the interaction between SMAC/Diablo can help ease the caspase IAPs mediated inhibition and promote apoptosis of cancer cells [20]. The above findings together with the results of the current study provide data support for regulating the expression of Smac to interfere with the apoptosis process and play a role in tumor suppression.

In conclusion, the present study found that Smac was lowly expressed in colon cancer. Increasing the expression of Smac inhibits the development of colon cancer, which may be related to inhibiting the expression of Survivin and promoting the expression of Caspase 3, then promoting the enhancement on promoting apoptosis function. This study provides an important direction for Smac to become a molecular targeted therapy for colon cancer, which needs to be further confirmed by future studies.

Conflict of Interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

This study was approved by the Animal Ethics Committee of Capital Medical University Animal Center.

Informed Consent

The authors declare that no patients were used in this study.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contributions

DZ and NY designed the study and performed the experiments, DZ collected the data, NY analyzed the data, DZ and NY prepared the manuscript. All authors read and approved the final manuscript.

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