

Original Research Article

Effects of SOCS3 on the development of colon cancer via regulation of HIF-1 α

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Abstract

Purpose: To investigate the influence of suppressor of cytokine signaling 3 (SOCS3) on rats with colon cancer (CC).

Methods: Sprague-Dawley (SD) rats were randomly divided into CC group and control group, and then CC rat model was constructed. The expression of SOCS3 in CC tissues was determined by quantitative real time-polymerase chain reaction (qRT-PCR). Hematoxylin-eosin staining (H&E) was used to examine colon tissue morphology. Immunohistochemistry (IHC) staining assay was performed to determine the expression of SOCS3 protein in colon tissues. The contents of HIF-1 α , phosphorylated phosphatidylinositol 3-hydroxy kinase (p-PI3K), and phosphorylated protein kinase B (p-AKT) proteins were determined by Western blotting (WB).

Results: Compared with that in the control group, the number of tumors in CC group was significantly increased ($p < 0.05$). 2). On the other hand, protein and message ribonucleic acid (mRNA) expressions of SOCS3 were down-regulated in CC group ($p < 0.05$). 3), while protein expressions of p-PI3K, p-AKT and HIF-1 α were raised in CC group ($p < 0.05$).

Conclusion: SOCS3 is lowly expressed in CC rats, and promotes the expression of HIF-1 α by activating PI3K/AKT signaling pathway. Thus, SOCS3 provides a therapeutic strategy for the management of colon cancer.

Keywords: Colon cancer, Suppressor of cytokine signaling protein 3 (SOCS3), Hypoxia inducible factor-1 α

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INTRODUCTION

Colon cancer (CC) is a malignant tumor commonly found in the digestive tract, with more than one-million new cases occurring each year [1]. The occurrence rate of CC presents striking

regional differences which vary between countries after age adjustments [2]. The incidence rate is highest in industrialized countries and lower in South America and China. The wide differences around the world are largely due to diet and other environmental factors [3].

Colon cancer is classified into well-differentiated CC, moderately and highly differentiated CC, and poorly differentiated CC, amongst which poorly differentiated CC accounts for about 20 % of CC, with poor prognosis [4]. Owing to the therapies which include surgery, chemotherapy, and radiotherapy, the 5-year survival rate of most actively treated patients is greater than 70 % in the early stage [5]. However, about 50 % of patients still suffer recurrence and metastasis, and die within 5 years. There is an alarming issue that CC patients tend to be younger, and the incidence rate of CC is steadily improving in individuals aged below 50 years old every year [6]. In recent years, great progress has been made in the pathogenesis and molecular mechanism of CC, but there is no effective molecular diagnostic method for predicting its prognosis. Therefore, exploring new molecular markers as early markers of the diagnosis, treatment and prognosis of CC is urgent.

Hypoxia and angiogenesis have a close relationship to cancer metastasis [7]. It has been found that many regulatory factors of pathological angiogenesis change under hypoxic conditions, causing tumor metastasis [8]. In the case of CC, hypoxia strengthen its aggressiveness, increasing the drug resistance and promoting the metastasis of the tumor, which is detrimental to the prognosis of patients. Hypoxia inducible factor-1 α (HIF-1 α), a factor usually induced under hypoxic conditions, regulates the expression of various cytokines such as vascular endothelial growth factor-A (VEGF-A), and plays a crucial role in angiogenesis and metastasis in cancer [9]. It has been shown in *in vitro* experiments that HIF-1 α affects the angiogenesis and metastasis of colorectal cancer cells [10]. However, the molecular mechanism and clinical data of the relationships of HIF-1 α with the prognosis and metastasis of CC are still controversial.

Suppressor of cytokine signaling 3 (SOCS3), a member of the SOCS family, is structurally composed of different functional domains including the N-terminal kinase inhibitory region, the central SH2 domain, and the C-terminal homology region (SOCS box) [11]. It has been reported that the expression of SOCS3 is suppressed in many human tumors due to aberrant methylation in its promoter region [12]. SOCS3 restores gene function through demethylation, and suppresses the growth of tumor cells by curbing signal transducer and activator of transcription 3 (STAT3) activation through gene transfection in several human cancer cells [13]. However, the mechanism by which SOCS3 regulates other intracellular signal

transduction cascades [such as phosphatidylinositol 3-hydroxy kinase (PI3K)/protein kinase B (AKT)] except for STAT3 is still poorly understood. The PI3K/AKT also plays a key role in tumorigenesis by affecting biological characteristics such as angiogenesis, migration and diffusion of malignant tumors. The PI3K/AKT pathway is crucial for multiple cellular functions including metabolism, proliferation, growth and survival [14]. There is increasing evidence indicating that this pathway is often dysregulated during tumorigenesis, thereby influencing certain biological phenotypes. In this study, the effects of SOCS3 on the expression of HIF-1 α and CC rats were investigated, in addition to whether the PI3K/AKT signaling pathway is involved in these processes.

EXPERIMENTAL

Establishment of rat models of CC

A total of 40 Sprague-Dawley (SD) rats weighing about 200 g (half male and half female) were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) and divided into control group and CC group. Next, rat models of CC were prepared according to the experimental requirements. The rats in CC group were subcutaneously injected with DMH at a dose of 30 mg/kg once every two days for 12 consecutive weeks, while those in Control group were injected with an equivalent volume of normal saline. The rats in both groups were fed with normal diet and water. This study was approved by the Animal Ethics Committee of Yantai Hospital of traditional Chinese Medicine Animal Center (approval no. 18-002). All procedures were conducted in accordance with the 'Animal Research: Reporting in Vivo Experiments guidelines 2.0' [15].

Hematoxylin-eosin (H&E) staining assay

After model establishment, the colon tissues of rats were separated, fixed in neutral formalin solution for 24 h, dehydrated with alcohol gradient from low concentration to high concentration, embedded in paraffin, and sliced into 5- μ m sections using a microtome. Then, the sections were subjected to H&E staining (Boster, Wuhan, China), and colon tissue lesions were examined under a microscope.

Immunohistochemical (IHC) staining assay

The rat CC tissues were prepared into paraffin sections. Next, the sections were subjected to IHC staining to determine the expression of the protein SOCS3 in tumor tissues. The paraffin

sections were deparaffinized, incubated in 3 % H₂O₂ for 30 min to inactivate endogenous peroxidase, rinsed with distilled water, washed with phosphate-buffered saline (PBS), and blocked with 5 % goat serum (dissolved in PBS) for 1 h. Thereafter, the blocking solution was discarded, and the sections were incubated with anti-SOCS3 (Santa Cruz, Santa Cruz, CA, USA) antibodies at room temperature overnight. Thereafter, the sections were washed and then incubated with horse radish peroxidase (HRP)-conjugated IgG secondary antibody at room temperature for 1 h, followed by washing with PBS and color development with diaminobenzidine (DAB) chromogen (Solarbio, Beijing, China). Then, the sections were counterstained with hematoxylin nuclear staining solution to reveal the nuclei, and then examined by a light microscope (Olympus, Tokyo, Japan).

Quantitative real time-polymerase chain reaction (qRT-PCR)

Total ribonucleic acids (RNAs) were isolated from tissue samples with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed into complementary deoxyribose nucleic acids (cDNAs) using a PrimeScript™ RT-PCR kit (TaKaRa, Dalian, China). Next, qRT-PCR was conducted using a Light Cycler 480II system (Roche, Basel, Switzerland) with SYBR Premix Ex Taq™. With glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference, gene expressions were standardized. The relative expression levels of all genes were measured with the 2^{-ΔΔCT} method. The sequence of primers used are shown in Table 1.

Determination of relevant protein levels by Western blotting (WB)

The tissues were lysed with RIPA lysis buffer (Roche, Basel, Switzerland) containing a protease inhibitor (PMSF) mixture on ice, and centrifuged at 14,000 rpm for 20 min. Then, the supernatant was collected, and the protein concentration of each sample was quantified using bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). Next, total proteins (30-50 μg) were separated via 10% sodium dodecyl

sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Billerica, MA, USA). Thereafter, the membrane was blocked in 5 % skim milk for 1 h and then incubated with the following specific primary antibodies at 4 °C overnight: β-actin, phosphatidylinositol 3-hydroxy kinase (PI3K) and phosphorylated PI3K (p-PI3K) (Abcam, Cambridge, MA, USA), and AKT and phosphorylated (p-AKT) (Cell Signaling Technology, Danvers, MA, USA). The next day, the membrane was incubated with the corresponding secondary antibodies, and specific bands were detected using SuperSignal West Pico chemiluminescent substrate (Thermo Fisher, Waltham, MA, USA).

Statistical analysis

SPSS statistical analysis software (version 26.0) was utilized for statistical analysis. Numerical variables are expressed as mean ± standard deviation (SD); independent samples *t*-test was adopted for comparison between two groups of data. *P* < 0.05 was considered statistically significant.

RESULTS

SOCS3 expression was downregulated in CC tissues

The number of colon tumors was significantly larger in the CC group than that in control group (*p*<0.01), while CC induction rate was 85 % in the CC group. The results of H&E staining assay showed that the Control group displayed normal ruddy mucosa, regular folds, normal glandular duct structure, and no obvious infiltration of inflammatory cells. In CC group, there were abnormally thickened mucosa, thickened and irregular folds, visible tumor nodules of various sizes throughout the entire colon, cancer cells of different sizes arranged in lines and clusters, massive infiltration of inflammatory cells and disappeared intercellular spaces. The messenger RNA (mRNA) expression level of SOCS3 in the CC tissues was first measured via qRT-PCR analysis.

Table 1: Primer sequences used in PCR

Indicator	Forward primer sequence (5' - 3')	Reverse primer sequence (5' - 3')
GAPDH	GGAGCGAGATCCCTCCAAAT	GGCTGTTGTCATACTTCTCATGG
PI3K	GCCCAGGCTTACTACAGAC	AAGTAGGGAGGCATCTCG
AKT	AGTCCCCACTCAACAACTTCT	GAAGGTGCGCTCAATGACTG
HIF-1α	ACCTTCATCGGAACTCCAAG	ACTGTTAGGCTCAGGTGAACT
SOCS3	GCTGGCGAAGGAAATGGT	GGAGCCTAGGGTGAAAGATG

The results revealed that the mRNA expression level of SOCS3 was significantly lower in the CC group than in the Control group ($p < 0.01$, Figure 1). Then, IHC staining assay was performed to identify SOCS3 protein in the CC tissues. It was discovered that the protein expression of SOCS3 was significantly decreased in the CC group ($p < 0.05$).

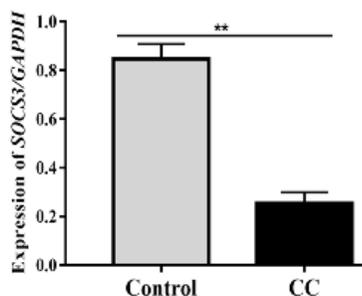


Figure 1: SOCS3 mRNA expression (qRT-PCR). Compared with that in the Control group, the mRNA expression of SOCS3 was reduced in CC group (** $p < 0.01$)

HIF-1 α expression was increased in CC group

The mRNA expression level of HIF-1 α in CC tissues was measured *via* qRT-PCR analysis, and it was discovered that the mRNA expression level of HIF-1 α was markedly higher in the CC group than that in the Control group ($p < 0.01$, Figure 2). Next, the protein expression of HIF-1 α in the CC tissues was determined with WB, and the results revealed that the protein expression of HIF-1 α rose in the CC group ($p < 0.05$, Figure 3).

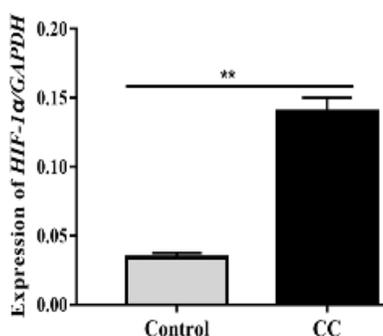


Figure 2: HIF-1 α mRNA expression (qRT-PCR). Compared with that in Control group, the mRNA expression of HIF-1 α rose in the CC group (** $p < 0.01$)

Changes in PI3K/AKT signaling pathway protein levels in CC rats

The results of qRT-PCR showed that the mRNA expressions of PI3K and AKT were clearly increased in the CC group when compared with

those in the Control group ($p < 0.01$, Figure 4). WB results demonstrated that the protein levels of p-PI3K and p-AKT were elevated in the CC group when compared with those in the Control group ($p < 0.05$, Figure 5).

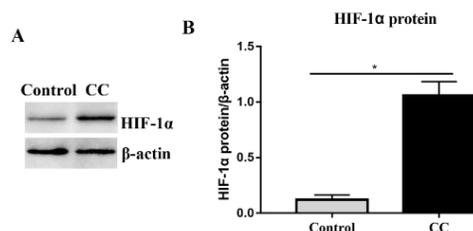


Figure 3: HIF-1 α protein expression. The protein expression of HIF-1 α was higher in CC group than that in control group; * $p < 0.05$

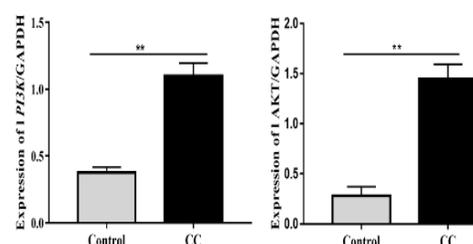


Figure 4: MRNA expressions of PI3K and AKT (qRT-PCR). Compared with those in the Control group, the mRNA expressions of PI3K and AKT rose in the CC group ($p < 0.01$)

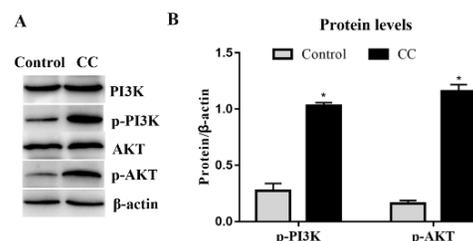


Figure 5: Protein expressions of p-PI3K and p-AKT (WB). The protein expressions of p-PI3K and p-AKT were higher in the CC group than those in the Control group; * $p < 0.05$

DISCUSSION

Oxygen supply is crucial for cell growth, and a tumor microenvironment characterized by hypoxia and nutritional deficiencies results in genetic and epigenetic adaptation of cells. It has been proven that hypoxic conditions enhance the motility and invasiveness of tumor cells. The adaptability of tumor cells to hypoxia makes them resistant to treatment. The adaptability of gene products to hypoxia is also important. There is evidence that most of these hypoxia-regulated genes are mediated by HIF-1 α . HIF-1 α

participates in hypoxia through oxygen homeostasis, and is also involved in myocardial, cerebral and retinal ischemia, pulmonary hypertension, preeclampsia, intrauterine growth, bradygenesis, and cancers. Besides, HIF-1 α plays a vital role in physiological homeostasis and pathological mechanisms. It is capable of promoting tumor progression by regulating the expression of various hypoxia-inducible genes, and often serves as a therapeutic target factor for cancers through modulation by growth factors involved in tumor progression. It has been confirmed that HIF-1 α is highly expressed in CC tissues to maintain its high proliferation rate and facilitate the metastasis of CC cells. Moreover, the increase in cell proliferation rate may in turn elevate the expression of HIF-1 α . The HIF-1 α silencing in CC cells inhibits their proliferation and promotes their apoptosis. Thus, HIF-1 α may be an effective therapeutic target for CC, and inhibition of HIF-1 α expression may be an appropriate method for developing molecule-targeted therapies.

A previous study demonstrated that SOCS3 significantly repressed the proliferation of lung cancer cells *in vitro*, and suggested that SOCS3 can act as a tumor suppressor gene in tumor development [12]. In addition, SOCS3 is capable of regulating the movement and migration of tumor cells. A previous report demonstrated that exogenous SOCS3 suppressed the progression of malignant fibrous histioma [16]. Moreover, SOCS3 widely serves as a miRNA target due to its abnormal expression in cancer cells and potential anti-tumor function. SOCS has been verified to be a key negative regulator of JAK/STAT signal transduction, which is critical in many immune and pathological processes. Among the eight family members, SOCS1 and SOCS3 are the most effective inhibitors of the JAK/STAT signaling pathway.

The activation of JAK/STAT signal transduction is necessary for cell transformation mediated by several oncogenes, inhibitory function of SOCS proteins needs to be overcome during the tumorigenesis of specific cells [17]. In addition to STAT, other intracellular signal transduction cascades, including Ras/ERK1/2 and PI3K/AKT, have also been proven in numerous studies to be regulated by SOCS3, and their significant and persistent activation is associated with tumorigenesis [13]. A study reported that SOCS3 regulates HIF-1 α expression by targeting AKT, thereby inhibiting the proliferation and angiogenesis in human small-cell lung cancer [18].

Previous reports have denoted that SOCS3 is

differentially expressed in CC tissues, but no studies have indicated that SOCS3 regulates HIF-1 α expression through the PI3K/AKT signaling pathway to alter the hypoxia state of tumor microenvironment in CC. In this study, it was found that SOCS3 was lowly expressed, HIF-1 α expression was increased, and the PI3K/AKT signaling pathway was activated in the CC rats. The mechanism of PI3K/AKT pathway alteration needs to be further explored. In this study, it seems that SOCS3 may regulate HIF-1 α expression through PI3K/AKT pathway in CC tissues, which not only enhances the regulatory mechanism of SOCS3 in the CC, but also lays a foundation for further investigation of the function of PI3K/AKT signaling pathway in CC. To the best of our knowledge, this is the first study that shows SOCS3 regulates HIF-1 α expression via PI3K/AKT signaling pathway in CC tissues.

CONCLUSION

SOCS3 is lowly expressed in CC rats and promotes the expression of HIF-1 α by activating PI3K/AKT signaling pathway. Thus, SOCS3 may play role in developing a new therapeutic approach to the management of colon cancer.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Fang Chen and Yifan Long contributed equally to this work.

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