Downregulation of Foxc2 enhances apoptosis induced by 5-fluorouracil through activation of MAPK and AKT pathways in colorectal cancer

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Abstract. The chemotherapy drug 5-fluorouracil (5-FU) is fundamental for the treatment of colorectal cancer (CRC); however, drug resistance to 5-FU may occasionally occur. Abnormal expression of Forkhead box C2 gene (Foxc2) has been identified in several human cancers, but the role of Foxc2 in the progression of CRC remains unclear. The present study established a stable Foxc2-shRNA cell line, which was confirmed by western blot analysis and quantitative polymerase chain reaction. The Foxc2-shRNA cells were treated with 5-FU and the cell viability was determined by an MTT assay. Western blot analysis was performed to investigate the signaling pathway involved in 5-FU treatment. The present study identified that 5-FU increased the percentage of apoptotic CRC cells among the Foxc2/RNA interference-transfected cells compared with cells transfected with an empty vector. Therefore, the downregulation of Foxc2, induced by 5-FU, may enhance apoptosis by the downregulation of apoptotic factors, including B cell lymphoma-2 and pro-caspase-3, in Foxc2-shRNA CRC cells. Furthermore, the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinases/protein kinase B (PI3K/AKT) pathways were essential for the sensitization effect of Foxc2 to 5-FU treatment. Overall, these findings reveal the mechanisms behind Foxc2 depletion and 5-FU treatment of CRC and suggest that Foxc2 enhances resistance to apoptosis, induced by 5-FU, through the activation of MAPK and PI3K/AKT pathways, and may serve as a valuable clinical prognostic marker for CRC.

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-associated mortality and morbidity in the USA, accounting for 9% of novel cases and mortalities that affect men and women (1). At present, the available diagnostic methods for CRC, including colonoscopy and fecal occult-blood test, remain unsatisfactory (2), and common premalignant lesions, such as sessile serrated adenomas, are extremely challenging to identify. The 5-year survival rate for patients with early stage CRC is ~80%, following radical surgery (2). In addition, during the process of CRC cell apoptosis induced by chemotherapy, tolerant cells often appear and escape treatment. Therefore, novel effective targets for anti-CRC treatment are required.

The forkhead box C2 (Foxc2) gene was first identified in mice during embryogenesis (3). The gene was then observed at high expression levels in a variety of malignancies, including esophageal (4), breast (5) and prostate (6) cancers. In addition, there is evidence that suggests that Foxc2 is associated with the regulation of cell proliferation, the development of angiogenesis (7) and the metastasis of human tumors (8-10). Previous studies have established that Foxc2 plays a predominant role in the modulation of cancer cells, which resist apoptosis-based tumor surveillance and treatments (11). Petrova et al reported that Foxc2 is highly expressed in developing lymphatic vessels and lymphatic valves (12). However, the functional role of Foxc2 has not yet been investigated under physiological and pathological conditions.

A preparatory study revealed that Foxc2 expression is significantly associated with the progression of CRC in vitro and in vivo, using reverse transcription-polymerase chain reaction (RT-PCR) and western blot analysis. This result is also demonstrated in CRC samples in paraffin-embedded tissues (13). Foxc2 was expressed in CRC patients and upregulated in the majority of these patients. Furthermore, the downregulation of Foxc2 lead to the apoptosis of cancer cells. Based on these results, the present study hypothesizes that the ablation of Foxc2 expression may lead human cancer cells, including CRC cells, to become sensitive to chemotherapy.

The present study, to the best of our knowledge, is the first to demonstrate that the morphological alterations observed...
in 5-fluorouracil (5-FU)-induced apoptosis are paralleled by Foxc2 deregulation. These results may have implications for the design of chemotherapy treatment; the combination of 5-FU treatment and Foxc2 depletion may lead to an improved treatment strategy for CRC patients.

Materials and methods

Cell lines. The study was approved by the Ethics Committee of Southern Medical University (Guangzhou, Guangdong, China). The human colon carcinoma HCT116 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). These cells were grown in Gibco RPMI-1640 media (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and supplemented with 10% fetal bovine serum (GE Healthcare, Life Sciences, Chalfont, UK) and 100 units Invitrogen penicillin-streptomycin (Thermo Fisher Scientific, Inc.) at 37°C, with a 5% CO₂ atmosphere in a humidified incubator.

Retroviral infection and reverse transcription. The Foxc2 expression construct was generated by cloning PCR-amplified, full-length or deletion mutant human Foxc2 cDNA into a pBabe plasmid (Addgene, Inc., Cambridge, MA, USA). RNA was extracted from the HCT116 cells using Trizol reagent (Thermo Fisher Scientific, Inc.), as previously described (14). Foxc2 cDNA was obtained using reverse transcription, which was performed using the Invitrogen SuperScript™ First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The human shRNA sequence, as described by Mani et al (15), was 5'-CCACAGTTTGCACACCAAGTTAC-3'. To knockdown Foxc2, the sequence was cloned into a pSUPER.retro.neo vector (Oligoengine, Seattle, WA, USA). Retroviral production and infection were performed, as previously described (16).

Quantitative PCR (qPCR) and western blot analysis. qPCR and western blot analysis were performed to confirm the successful infection of the CRC cells. qPCR was performed using the Applied Biosystems ABI PRISM® 7500 Sequence Detection System (Thermo Fisher Scientific Inc.) and the iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc.). The following primary rabbit anti-mouse monoclonal antibodies (BD Biosciences, San Diego, CA, USA) were used: Anti-α-tubulin rabbit anti-mouse monoclonal antibody (dilution, 1:2000; catalog no. KM9007; Tianjin Sungene Biotech Co., Ltd., Tianjin, China) was used as an internal loading control. Following washing with TBS-T, the nitrocellulose membrane was incubated with a secondary antibody against rabbit immunoglobulin (Ig)G or mouse IgG. The membrane was examined using an FluorChem FC2 Imaging System (Alpha Innotech, San Leandro, CA, USA), according to the manufacturer's protocol.
Statistical analysis. Statistical analyses were performed using SPSS version 13.0 software (SPSS, Inc., Chicago, IL, USA). Comparisons between groups for statistical significance were performed using a two-tailed paired Student's $t$ test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Establishment of Foxc2 small hairpin (sh) RNA stable cell lines. Foxc2-shRNA stable cell lines were established to study the role of Foxc2 in CRC cell survival. Western blot analysis and RT-qPCR revealed that protein and mRNA expression of Foxc2 was markedly downregulated in HCT116-shFoxc2 cells, in contrast to those in HCT116-vector cells (Figs. 1 and 2).

Ablation of Foxc2 expression leads to human colon cancer HCT116 cell lines becoming sensitized to 5-FU. An MTT assay was performed to examine the effect of 5-FU on the survival rate of Foxc2-knockdown cells. The cells were treated with 5-FU for 48 and 72 h, and it was demonstrated that downregulation of Foxc2 significantly decreased the growth rate of HCT116 cells compared with vector cells. In detail, the present study identified that the viability of the HCT116-Foxc2/RNA interference cells were decreased compared with HCT116-vector cells (control). Following treatment with 5-FU for 48 h, the half maximal inhibitory concentration (IC$_{50}$) of 5-FU in the HCT116-vector and HCT116-shFoxc2 cells were 6.3919 mg/l and 5.2965 mg/l ($P>0.05$), respectively. Following treatment for 72 h, the IC$_{50}$ decreased to 1.8839 and 1.1238 mg/l, respectively ($P<0.05$; Fig. 3; Table I). The results suggest that following a depletion of Foxc2 all the cells become sensitized to 5-FU, particularly following a 72-h treatment time.

Foxc2 aids cells in resisting apoptosis and regulating the expression levels of Bcl-2, Bax and caspase-3. Due to the unsatisfactory clinical therapies available, as a result of the resistance of cancer cells to apoptosis, the present study investigated whether Foxc2 enhanced the anti-apoptotic activity of CRC cells. To investigate the mechanism by which apoptosis is upregulated when treated with 5-FU and a knockdown of Foxc2, the expression levels of the apoptosis regulators Bcl-2, Bax, pro-caspase-3 and cleaved-caspase-3 were observed in HCT116-shFoxc2 and HCT116-vector cells. Western blot analysis revealed a downregulation of Bcl-2 and pro-caspase-3 in Foxc2-knockdown cells, while Bax and cleaved-caspase-3 were upregulated (Fig. 4).

MAPK and PI3K/AKT pathways are essential for the sensitization effect of Foxc2 to 5-FU treatment. The PI3K/AKT signaling pathway is an important survival pathway in numerous cellular systems and the activation of this pathway is required to prevent cell apoptosis (19,20). To investigate whether the depletion of Foxc2 enhances the apoptosis of cells through the PI3K/AKT pathways, the present study analyzed p-AKT and total AKT levels. As revealed in Fig. 5, p-AKT was decreased in HCT116-shFoxc2 cells, but the total AKT levels were not

Table I. Data obtained from the MMT assay, which examined the effect of 5-fluorouracil on the survival of Foxc2 knockdown cells.

| Variable | HCT116-vector, control | HCT116-Foxc2/RNAi cells |
|----------|------------------------|-------------------------|
|          | Mean                   | SD                      | Mean                   | SD                      |
| Data set 1 |                        |                         |                        |                         |
| 0.5      | 0.149116600            | 0.096946720             | 0.133288900            | 0.034042870             |
| 1        | 0.277738500            | 0.062077910             | 0.404865100            | 0.025722880             |
| 2        | 0.327444000            | 0.062361180             | 0.377874000            | 0.067800570             |
| 4        | 0.407302700            | 0.126097800             | 0.486171300            | 0.033699700             |
| Data set 2 |                        |                         |                        |                         |
| 0.5      | 0.420335700            | 0.059749480             | 0.473311200            | 0.054933290             |
| 1        | 0.583677900            | 0.074168170             | 0.637435200            | 0.021126600             |
| 2        | 0.620165400            | 0.042991280             | 0.757917100            | 0.014314910             |
| 4        | 0.618219400            | 0.068660390             | 0.811879100            | 0.009069179             |

Foxc2, forkhead box C2 gene; HCT116, human colon cancer HCT116 cell line; RNAi, RNA interference; SD, standard deviation.
altered. The result confirms that the PI3K/AKT pathway plays an important role in the sensitization effect of Foxc2 to 5-FU treatment.

In addition, in the MAPK pathway, the present study observed that although the total levels of ERK and JNK were not significantly altered subsequent to treatment with 5-FU, p-ERK did markedly decrease with the downregulation of Foxc2. By contrast, the levels of p-JNK increased, indicating Foxc2-mediated activation may occur via the MAPK pathway.

**Discussion**

Previous studies have demonstrated that Foxc2 is vital in tumor metastasis and metabolism (21). The present study revealed that the development of CRC is associated with a high expression of Foxc2, and a downregulation of Foxc2 enhanced the apoptotic rate of CRC cells. These findings indicate that Foxc2 may act as a potential diagnostic marker in CRC, and a specific inhibitor of Foxc2 may be a novel strategy for the treatment of CRC patients.

Resisting the apoptosis of cells is a hallmark of the majority of cancers (22). At present, the elimination of the resistance to apoptosis is an anticancer therapy (23). Activation of apoptotic
pathways is currently considered a vital step in the development of tumors (24,25). Therefore, the identification of the mechanism underlying the apoptotic pathway is important. Common stresses inducing apoptosis are imbalances in signaling pathways, resulting in altered levels of oncogene signaling. Alternatively, tumors may increase the expression of anti-apoptotic regulators, including Bcl-2 and Bcl-extra large, and survival signals, such as insulin-like growth factor 1, by downregulating pro-apoptotic factors, including Bax, Bcl-2 and cleaved-caspase-3, or by eluding the extrinsic ligand-induced death pathway (26).

The present study demonstrated that Foxc2 is vital in creating an anti-apoptotic environment in CRC cells that is relatively insensitive to chemotherapy. The increased expression of Foxc2 in CRC cells enhances the resistance to apoptosis induced by 5-FU, a common chemotherapeutic drug used in alimentary canal neoplasm. By contrast, a knockdown of Foxc2 markedly enhanced the sensitivity of the cells to 5-FU; therefore, allowing the cells to undergo apoptosis. Therefore, Foxc2 may be a potential target for chemotherapy.

MAPK and PI3K/AKT signaling pathways are frequently involved in the promotion of proliferation of cells (20,27,28), while inhibition of the MAPK pathway suppresses the proliferation of cells by induction of cell apoptosis through caspase activation (29). The activation of several proteins, including ERK, JNK and AKT, is regulated by the MARK and PI3K/AKT pathways (30-32). The present study investigated whether PI3K and MAPKs are critical in the Foxc2 inhibition of apoptosis, and demonstrated that several MAPK-regulated proteins were upregulated in Foxc2-overexpressing CRC cells and downregulated in Foxc2-inhibited CRC cells. Since Foxc2 is a transcriptional gene, it may not affect the MAPK and PI3K/AKT pathways through phosphorylation directly, and it is evident that the molecular mechanism of how Foxc2 affected the pathway requires additional investigation. Furthermore, several other questions require resolution; the potential role of Foxc2 in CRC cells remains unclear, and the involvement of other signaling pathways in the anti-apoptosis of CRC should be determined, although the present study doubts the involvement of additional pathways. Consequently, additional details concerning the function of Foxc2 require investigation.

In conclusion, the present study demonstrates the methods behind the combination of Foxc2 depletion and 5-FU treatment. A knockdown of Foxc2 induces cancer cells to become more sensitive to 5-FU, and a depletion of Foxc2 enhances 5-FU-induced apoptosis. Furthermore, the present study demonstrated that the MAPK and PI3K/AKT pathways are critical for the development of Foxc2. In the present study, a knockdown of Foxc2 inhibits AKT activation and regulates the expression of Bcl-2 and Bax. In addition, p-ERK markedly decreased with the downregulation of Foxc2 under the treatment with 5-FU. Overall, the present study reveals that a combination of Foxc2 depletion and 5-FU treatment may be a potential clinical therapy for CRC.

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