Original Article

IGFBP2 promotes the EMT of colorectal cancer cells by regulating E-cadherin expression

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Abstract: This study aimed to investigate the expression of insulin like growth factor binding protein 2 (IGFBP2) in colorectal cancer cells and its effect on the biological characteristics of cancer cells. We first established IGFBP2 knockdown (HCT116-shIGFBP2) and overexpression (HT29-IGFBP2) cell lines. Western blotting was used to evaluate the overexpression and knockdown efficiency. Next, the effect of IGFBP2 on colorectal cancer cell proliferation and migration was evaluated through cell proliferation and wound healing assays, respectively. Cell proliferation experiments showed that the upregulation of IGFBP2 promoted the proliferation of HT29 cells, but the downregulation of IGFBP2 inhibited the proliferation of HCT116 cells. Moreover, a wound healing assay showed that the migration ability of HCT116 cells was significantly reduced after the downregulation of IGFBP2. Also, the level of E-cadherin in HCT116-shIGFBP2 cells was significantly upregulated following IGFBP2 knockdown. Further analyses showed that colorectal cancer cells secreted high levels of IGFBP2 into the extracellular matrix, which inhibited E-cadherin expression as well. Overall, the results of this study showed that IGFBP2 inhibits the expression of E-cadherin and promotes the proliferation and migration of colorectal cancer cells.

Keywords: Colorectal cancer, insulin like growth factor binding protein 2 (IGFBP2), epithelial-mesenchymal transition (EMT), E-cadherin

Introduction

Colorectal cancer is one of the most common gastrointestinal malignancies and ranks third in cancer-related mortality in males and females worldwide [1]. Nowadays, the incidence and mortality rates of colorectal cancer in China are on the rise [2]. In recent years, due to the changes in people's dietary and living habits, morbidity and mortality due to colorectal cancer have risen rapidly. Colorectal cancer is highly aggressive, such that more than half of colorectal cancer patients often reach the stage at which radical resection of the tumor is impossible. Between 20% and 50% of colorectal cancer patients suffer from metastasis and die within 5 years of diagnosis [3, 4]. In recent years, although research on colorectal cancer has made significant progress in early diagnosis and treatment, the five-year survival rate of colorectal cancer patients is still low. Therefore, further understanding of the molecular mechanisms underlying the development of colorectal cancer is of great significance in finding new therapeutic targets that can inhibit tumor metastasis and improve the prognosis of patients.

The insulin-like growth factor (IGF) system is comprised of IGF, IGF receptors, and a family of IGF binding proteins (IGFBPs). As it plays important roles in growth and development, each component of this system is critical to the maintenance of normal homeostasis. IGFBP2 is a member of the IGFBP family, which can regulate the function of IGF [5]. IGFBP2 regulates a variety of cell activities through its downstream target molecules, playing an important role in cell proliferation, invasion, angiogenesis, and apoptosis. IGFBP2 exhibits a variety of complex functions in different cell types and cell micro-environments. Recent studies have shown that the high expression of IGFBP2 is also closely related to the occurrence of a variety of tumors [6, 7]. However, the effect and mechanism through which IGFBP2 affects the biological...
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characteristics of colorectal cancer cells still need to be further studied.

In this study, the effects of IGFBP2 on the proliferation and migration of colorectal cancer cells and their underlying mechanisms were investigated.

Materials and methods

Cell culture and transfection

The colon cancer cell lines, HT29 and HCT116, were cultured at 37°C in Mccoys'5A medium containing 10% fetal bovine serum, in a humidified incubator containing 5% CO₂. HCT116 cells were transfected with IGFBP2-shRNA (shIGFBP2) (Gene Pharma, Shanghai, China) and an empty vector, while HT29 cells were transfected with IGFBP2 and an empty vector. After 6 h, the transfection media were replaced with normal media, and the transfected cells were cultured for a further 24 h. The transfected cells were selected in a medium containing puromycin.

Cell proliferation assay

An MTT assay was used (according to the manufacturer's instructions) to detect cell proliferation. First, 2 × 10^3 cells were seeded in 96-well culture plates. After 24 h, MTT was added to each well and the plate was incubated at 37°C for an additional 4 h. Subsequently, the media was replaced with dimethylsulfoxide (DMSO), and the samples were shaken for 15 min to dissolve the formazan crystals. A microplate spectrophotometer was used to measure absorbance at 490 nm. Each MTT assay was repeated at least three times.

Wound healing assay

A wound healing assay was used to detect cell migration. A line was drawn at the center of the bottom of the 6-well plate with a marker pen in advance. The cells in the logarithmic growth phase were digested and resuspended, and then inoculated, in 6-well plates at 37°C in Mccoys'5A medium containing 10% fetal bovine serum, in a humidified incubator containing 5% CO₂. The inoculation density was up to 40-50%, which led to a cell density of 80-90% the next day. After incubation for 24 h, a 200 μL sterile pipette was used to draw scratches along the 6-well culture plate perpendicular to the marking line. The floating cells were washed using PBS, and the cells were cultured at 37°C in Mccoys'5A medium containing 0.1% fetal bovine serum, in a humidified incubator containing 5% CO₂. The scratch widths were observed and recorded under a microscope at 0 h, 12 h, 24 h, and 36 h, respectively.

Western blot

The cells were initially lysed in a 1 × SDS loading buffer containing protease inhibitors. Next, 30 μg of cell lysates were subjected to SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then incubated with specific antibodies for β-actin (1:10^4, Sigma), IGFBP2 (1:3000, Sigma), E-cadherin (1:3000, Sigma), and visualized using a SmartChem™ Image Analysis System (Beijing Sage Creation Science, China).

Statistical analysis

All data were analyzed using the statistical package SPSS 23.0, and the data were presented as the mean ± SD. P values < 0.05 were considered statistically significant.

Results

IGFBP2 promotes cell proliferation in vitro

To investigate the effects of IGFBP2 on cell proliferation, its expression was knocked down using shRNA. As shown in Figure 1A, the expression level of IGFBP2 in the HCT116-shIGFBP2 cells was significantly reduced compared to the level in the control (empty vector) cells. Furthermore, the knockdown of IGFBP2 significantly inhibited cell growth (Figure 1B), as revealed by an MTT assay. To validate the effects of IGFBP2 on cell proliferation, IGFBP2 was overexpressed in the HT29 cell line. As shown in Figure 1C, the level of IGFBP2 in the HT29-IGFBP2 cells was significantly increased compared to the level in the control (empty vector) cells. Furthermore, the overexpression of IGFBP2 significantly promoted cell growth (Figure 1D) as revealed by the MTT assay.

IGFBP2 promotes cell migration in vitro

As HCT116 cells exhibited a higher migration ability, we conducted a wound healing assay of...
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Figure 1. The effect of IGFBP2 on cell proliferation. A. In the HCT116 cell line, the expression level of IGFBP2 in HCT116-shIGFBP2 cells was significantly reduced compared to the level in the control (empty vector) cells. B. In the HCT116 cell line, the knockdown of IGFBP2 significantly inhibited cell growth. C. In the HT29 cell line, the expression level of IGFBP2 in HT29-IGFBP2 cells was significantly increased compared to the level in control (empty vector) cells. D. In the HT29 cell line, the overexpression of IGFBP2 significantly promoted cell growth.

Both intracellular and extracellular IGFBP2 regulates the expression of E-cadherin

To investigate the mechanisms involved in the IGFBP2-knockdown-mediated inhibition of cell migration, western blotting was used to explore the effects of intracellular IGFBP2 on E-cadherin expression. As shown in Figure 3, the level of E-cadherin in the HCT116-shIGFBP2 cells was significantly upregulated following the IGFBP2 knockdown, compared to the level in the control (empty vector) cells.

Next, the protein secreted by the HCT116 cells in the extracellular culture medium (CM) was analyzed, and it was found that IGFBP2 was heavily secreted into the CM of both the HCT116-shIGFBP2 (HCT116-shIGFBP2-CM) and the control (empty vector) cells (HCT116-V-CM) (Figure 4).

The effect of extracellular IGFBP2 on E-cadherin expression was investigated by culturing HCT116 cells with HCT116-shIGFBP2-CM. Three different concentrations (5 µg/mL, 50 µg/mL, and 500 µg/mL) of IGFBP2 Ab were added to the HCT116-shIGFBP2-CM to neutralize extracellular IGFBP2. Cell lysates were collected after culturing HCT116 cells for 0.5 h, 1 h, 3 h, and 6 h. The E-cadherin expression in HCT116 cells was upregulated after they were cultured in HCT116-shIGFBP2-CM containing 500 µg/mL IGFBP2 Ab for 1 h (Figure 5).

Discussion

In recent years, due to changes in people’s dietary and living habits, the morbidity and mortality of colorectal cancer has risen rapidly. On the one hand, the symptoms of colorectal cancer mainly depend on the location of the tumor and the occurrence of metastasis, with general symptoms including rectal bleeding, diarrhea, constipation, weight loss, and abdominal pain [8]. On the other hand, due to the high metastatic invasiveness of colorectal cancer, more than 50% of patients often reach a stage at which radical resection of the tumor becomes impossible [9]. Although research on the early diagnosis and prevention of colorectal cancer metastasis has made some progress in recent years, the five-year survival rate of colorectal cancer patients still remains low. Therefore, further studies on the molecular mechanism of the occurrence and development of colorectal cancer are still needed to develop novel techniques and targets to inhibit tumor metastasis and improve the prognosis of patients.

The epithelial-mesenchymal transition (EMT) is a process during which polarized epithelial cells obtain mesenchymal phenotypes [10]. Through EMT, cells lose polarity and cell-cell adhesion and acquire migration and invasion abilities [10, 11]. Many studies have shown that EMT contributes to the invasiveness and pro-
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In recent years, several studies have shown that many malignant tumors show a high expression of IGFBP2, such as prostate cancer, ovarian cancer, and colon cancer, and IGFBP2 has been used as a prognostic biomarker for some tumors and leukemia [13, 14], indicating its important roles in the occurrence and development of tumors. Moreover, IGFBP2 has many cellular functions associated with tumor cell growth, adhesion, and invasion [15].

Unregulated proliferation and migration are important biological characteristics of malignant cells, which are also important reasons why malignant tumors become lethal. The invasion and metastasis of tumor cells into surrounding cell tissues are dependent on the invasion and metastasis ability of the tumor cells. Therefore, the detection of tumor cell invasion and metastasis, the prediction of tumor biological behavior, and the evaluation of treatment prognosis are of great significance. There are many studies demonstrating that IGFBP2 is involved in cancer cell proliferation, migration, and invasion. The mechanism of IGFBP2 in regulating metastasis in different tumors is slightly different. In colorectal cancer, IGFBP2 can activate neuronal adhesion receptor L1 and promote tumor metastasis [16]. In breast cancer, IGFBP2 promotes lymph node metastasis through synergistic β-catenin action [17]. In glioma cells, IGFBP2 promotes the formation of planar pseudopodia on the surface of tumor cells and reduces the formation of adhesive plaques, enhancing the motor ability of tumor cells and leading to metastasis [18]. IGFBP2 has also been reported to be significantly overexpressed in the serum and plasma.

Figure 2. The effect of IGFBP2 on cell migration. A Wound healing assay showed (A: Low power and B: High power) that the knockdown of IGFBP2 significantly inhibited cell migration.

Figure 3. The effect of intracellular IGFBP2 on E-cadherin expression. E-cadherin was significantly upregulated following IGFBP2 knockdown in the HCT116 cell line.

Figure 4. IGFBP2 was heavily secreted into the extracellular condition medium (CM) of both HCT116-shIGFBP2 (HCT116-shIGFBP2-CM) and the control (empty vector) cells (HCT116-V-CM).
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of colorectal cancer patients compared with normal controls [19-22]. IGFBP2 has been previously reported as a potential plasma marker for colorectal cancer [20].

In this study, the effect of IGFBP2 on the proliferation and migration of colorectal cancer cells was determined, and the underlying molecular mechanism was explored.

Our results showed that IGFBP2 affected both the proliferation and migration of colorectal cancer cells. The overexpression of IGFBP2 in HT29-IGFBP2 cells can promote cell proliferation, but the knockdown of IGFBP2 in HCT116 cells can inhibit cell proliferation. This finding is consistent with the results of previous studies [23, 24]; however, IGFBP2 can regulate cell proliferation and mediate tumor development through multiple signaling pathways, such as IGF/IGFR/IGFBP, PTEN/PI3K/Akt, ERK, and mitogen-associated protease [23-25]. The underlying molecular mechanism of the IGFBP2-mediated regulation of colon cancer cell proliferation remains to be further studied.

The results of this study also showed that the migration ability of HCT116 cells was significantly reduced after knocking down the IGFBP2 expression, suggesting that IGFBP2 knockdown inhibited the migration of colon cancer cells. As regulation of E-cadherin expression is a potential mechanism of EMT, we used fresh serum-free conditioned medium to culture cells to explore the effects of intracellular IGFBP2 on E-cadherin expression. The results showed that E-cadherin expression was upregulated after IGFBP2 knockdown, suggesting that intracellular IGFBP2 may promote EMT in colon cancer cells by regulating the expression of E-cadherin, and thus, enhancing their migration ability.

In addition, since IGFBP2 is secreted in large quantities in the extracellular matrix, we also measured the amount of IGFBP2 in the extracellular matrix, and the results showed that the extracellular CM of colorectal cancer cells contained a high amount of IGFBP2. We also found that extracellular IGFBP2 inhibits E-cadherin expression, and this effect of extracellular IGFBP2 in HCT116-shIGFBP2-CM can be neutralized by treatment with 500 µg/mL IGFBP2 Ab, as was observed with the upregulation of E-cadherin expression in HCT116 cells after they were cultured in HCT116-shIGFBP2-CM with 500 µg/mL IGFBP2 Ab for 1 h. However, we did not observe similar changes after treatment for 0.5 h, 3 h, and 6 h. One of the reasons for this observation could be that a 0.5 h culturing period could be too short for cells to exhibit the phenotype change of E-cadherin expression, but 3 h and 6 h culturing periods could be too long, and CM presented a bad environment.

**Figure 5.** The effect of extracellular IGFBP2 on E-cadherin expression. HCT116-shIGFBP2-CM was used to culture HCT116 cells. Three different concentrations (5 µg/mL, 50 µg/mL, and 500 µg/mL) of IGFBP2 Ab were added to the HCT116-shIGFBP2-CM to neutralize extracellular IGFBP2. Cell lysates were collected after culturing HCT116 cells for 0.5 h, 1 h, 3 h, and 6 h. The E-cadherin expression in the HCT116 cells was upregulated after they were cultured in HCT116-shIGFBP2-CM containing 500 µg/mL IGFBP2 Ab for 1 h.
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for cell survival. Another reason could be that IGFBP2 and IGFBP2 Ab are both proteins that are easily degraded in the absence of protection.

In conclusion, the function of IGFBP2 is complex and diverse. This study showed that IGFBP2 promotes the proliferation and migration of colorectal cancer cells via the inhibition of E-cadherin expression. However, current research on IGFBP2 is incomplete and still needs further work. We believe that, with further studies, the exact roles and specific mechanisms underlying the effect of IGFBP2 in tumor cells can be elucidated. IGFBP2 may be a candidate target for the treatment of colorectal cancer in the future.

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Disclosure of conflict of interest

None.

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