HMGB1 knockdown effectively inhibits the progression of rectal cancer by suppressing HMGB1 expression and promoting apoptosis of rectal cancer cells

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Abstract. Rectal cancer is a malignant gastrointestinal tumor, which is associated with high morbidity and mortality. High-mobility group protein 1 (HMGB1) is widely present in the nucleus of eukaryotic cells, and is highly conserved between humans and rodents. Recently, HMGB1 has been reported to be involved in the progression and metastasis of human cancer; however, its role in the development and metastasis of human rectal cancer remains unclear. The present study detected the expression levels of HMGB1 in pathological specimens from patients with clinically identified rectal cancer using immunohistochemistry and western blotting. The results demonstrated that HMGB1 was highly expressed in samples from patients with rectal cancer. The positive rate of HMGB1 in rectal cancer tissues was 96.08% (49/51), which was significantly higher compared with 3.92% (2/51) in normal tissues. In addition, western blotting indicated that HMGB1 was distributed and located not only in the nucleus, but also in the cytoplasm of colorectal cancer cells. HMGB1-specific short hairpin (sh)RNA was used to silence the endogenous expression of HMGB1 in colorectal cancer cells. A functional assay demonstrated that knockdown of endogenous HMGB1 expression significantly inhibited the proliferation of SW620 and Colo320 cells. Furthermore, western blotting revealed that knockdown of endogenous HMGB1 expression contributed to activation of caspase-3 and the substrate poly(ADP-ribose) polymerase. The expression levels of B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) were also detected by western blotting. As expected, decreased levels of Bcl-2 and increased levels of Bax were detected in the HMGB1 shRNA-transfected colorectal cancer cells, and the Bax/Bcl-2 ratio was increased in HMGB1 shRNA-transfected cells. These data indicated that HMGB1 may act as an oncogene in rectal cancer, and knockdown of endogenous HMGB1 expression may significantly inhibit the proliferation of colorectal cancer cells and promote apoptosis of tumor cells. Further research regarding the mechanisms underlying the effects of HMGB1 on the progression of rectal cancer may provide novel targets for the treatment of rectal cancer, and provide a theoretical reference for clinical treatment.

Introduction

Rectal cancer is one of the most common types of gastrointestinal malignancy, and refers to tumors that occur between the dentate line and the rectosigmoid junction (1). The median age of patients with rectal cancer is ~45 years; however, the incidence in young people has begun to increase. Rectal cancer is easily diagnosed by a digital rectal examination and sigmoidoscopy (2-4). However, since the cancer is located deep within the pelvic cavity, and due to the key anatomical differences between the rectum and colon, the management and treatment of rectal cancer is significantly difficult, particularly in the curative setting (5). Rectal tumors are not easily completely removed by surgery alone, thus leading to a high recurrence rate in patients with rectal cancer (6), which is a common cause of cancer-associated mortality worldwide. Although great progress has been made regarding the diagnosis and treatment of rectal cancer, the prognosis remains poor for patients with distant metastasis of rectal cancer (7). Clinically, micrometastases occur in the majority of patients with rectal cancer prior to undergoing radical operation, and are the direct cause of metastasis and recurrence post-surgery in patients with rectal cancer (2,5). Therefore, it is essential to further explore the mechanisms underlying the development and progression of rectal cancer and to clarify its biological characteristics, in order to develop more effective therapeutic targets that may improve the prognosis of patients with rectal cancer.

High-mobility group protein 1 (HMGB1) is a recently discovered oncogene, with a molecular weight of ~30 kD (8). HMGB1 is a non-histone chromosomal protein, which is widely present in the nucleus of eukaryotic cells. It is named for its ability to exist in the nucleus of eukaryotic cells, and is highly conserved between humans and rodents. Although great progress has been made regarding the diagnosis and treatment of rectal cancer, the prognosis remains poor for patients with distant metastasis of rectal cancer (7). Clinically, micrometastases occur in the majority of patients with rectal cancer prior to undergoing radical operation, and are the direct cause of metastasis and recurrence post-surgery in patients with rectal cancer (2,5). Therefore, it is essential to further explore the mechanisms underlying the development and progression of rectal cancer and to clarify its biological characteristics, in order to develop more effective therapeutic targets that may improve the prognosis of patients with rectal cancer.

Key words: rectal cancer, high-mobility group protein 1, cell apoptosis, caspase-3

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fast migration velocity in polyacrylamide gel electrophoresis (PAGE) (9,10). HMGB1 is regarded as a proinflammatory factor that is secreted from the nucleus to the extracellular space. In addition, HMGB1 is highly conserved between humans and rodents, and the amino acid sequences are >99% homologous. Notably, the amino acid sequence of HMGB1 is exactly the same between rats and mice. HMGB1 has two DNA-binding motifs: A box and B box, which execute two major functions. The HMGB1 B box exerts cytokine activity, whereas a box has an inverse role and inhibits the activity of native HMGB1. HMGB1 has various receptors, including receptor for advanced glycation end products (RAGE), Toll-like receptors (TLRs), syndecan and plasminogen, among which, RAGE and TLR4/TLR2 are the main receptors of HMGB1.

In recent years, high expression levels of HMGB1 have been detected in breast cancer (11), colon cancer (12,13), lung cancer (14,15), bladder cancer (16), ovarian cancer (17), and other tumors (18). HMGB1 is involved in numerous processes during tumor formation and development, including unlimited cell proliferation, angiogenesis, programmed cell death evasion, and resistance to growth factor inhibitors. Previous studies have reported that elevated expression of HMGB1 is closely associated with a high incidence of tumor invasion and metastasis (19-21). However, the role of HMGB1 in human rectal cancer remains to be clarified. The present study detected the expression levels of HMGB1 in rectal cancer specimens and explored the mechanisms underlying the effects of HMGB1 on colorectal cancer cells. Understanding the mechanisms underlying rectal cancer may help to identify novel targets during the progression and metastasis of rectal cancer.

Materials and methods

Patients. A total of 51 patients with rectal cancer and 51 normal controls were included in the present study. In the rectal cancer group, 39 patients were male (76.5%) and 12 patients were female (23.5%). In the control group, 32 patients were male (62.7%) and 19 patients were female (37.3%). The median age was 60.47 years, with a range of 26-85 years. The present study was approved by the ethics committee of Qingdao Central Medical Group (Qingdao, China), and all patients provided written informed consent. The subjects were well informed of the details of the study and signed relevant contracts prior to the study. Half of each postoperative rectal specimen was immediately fixed in 10% neutral buffered formalin solution and embedded in paraffin. The other half of each specimen was frozen and maintained at -80°C until further use. The expression levels of HMGB1 were detected by immunohistochemical analysis and western blotting. Peritumoral tissue was frozen and maintained at -80°C until further use. The other half of each specimen was immediately fixed in 10% neutral buffered formalin solution and embedded in paraffin. The other half of each specimen was frozen and maintained at -80°C until further use. The expression levels of HMGB1 were detected by immunohistochemical analysis and western blotting. Peritumoral tissue was frozen and maintained at -80°C until further use.

Cell lines and agents. The SW620 and HCT116 colorectal cancer cell lines were obtained from the Chinese Academy of Tumor Cell Collection (Manassas, VA, USA), and Colo320 colorectal cells were purchased from the Chinese Academy of Tumor Cell Bank (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both HyClone; GE Healthcare Life Sciences, Logan, UT, USA) at 37°C with 5% CO₂. The human HMGB1 short hairpin (sh)RNA and control shRNA (cat. no. TR30013) were obtained from Origene Technologies, Inc. (Rockville, MD, USA). The HMGB1 shRNA consisted of four unique 29mer shRNAs (cat. no. TG316576). Lipofectamine 2000 was used for transfection of the cells (Invitrogen; Thermo Fisher Scientific, Inc, Waltham, MA, USA).

Antibodies and small interfering (si)RNAs. Mouse monoclonal anti-HMGB1 (cat. no. ab77302) was obtained from Abcam (Cambridge, MA, USA). Rabbit polyclonal anti-caspase-3 (cat. no. 3138-100) was purchased from BioVision, Inc. (Milpitas, CA, USA). Rabbit polyclonal anti-B-cell lymphoma 2 (Bcl-2; cat. no. API303a-ev), mouse polyclonal anti-Bcl-2-associated X protein (Bax; cat. no. API302a-ev) and mouse monoclonal anti-β-actin (cat. no. P60709) were obtained from Abgent Biotech Co., Ltd. (Suzhou, China), and anti-cleaved-PARP (cat. no. ab4830; Abcam, Cambridge, MA, USA). All antibodies were used at a dilution of 1:1,000. The siRNAs were designed and synthesized by GenePharma Corp. (Shanghai, China). The HMGB1-specific siRNA sequences were as follows: HMGB1 siRNA1, sense 5'-GGAGAGCUCA AAGAGCCGATT-3', antisense 5'-UCGGCUUCUUAGGAU GCCCTT-3'; HMGB1 siRNA2, sense 5'-CUCCGAAGCUGA AGGAAAATT-3', antisense 5'-UUUUCUCUGCACGUUC GCAGTT-3'; and HMGB1 siRNA3, sense 5'-GGGAGGAGC AUAGAGAATT-3', antisense 5'-UUUCUCUUAGCUCC UCCCTT-3'. Negative control (N.C.) siRNA was purchased from GenePharma Corp. (cat. no. A01004), which was purified by HPLC and the unpaired single chains were truly removed.

Transfection. The SW620 cells (1x10⁶ cells/well) were plated into a 48-well plate. After 8 h, 200 ng of three pairs of HMGB1-specific siRNA and 2 µl Lipofectamine 2000 (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The mixture was added into the medium of human colorectal cancer cells and cultured for 48 h.

MTT assay. A 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to determine the proliferation of SW620 and Colo320 cells. The SW620 and Colo320 colorectal cancer cells were plated into 48-well plates at a density of 2x10⁴ cells/well. After 6 h, the cells were transfected with HMGB1 shRNA or N.C. shRNA using Lipofectamine 2000, and the cells were cultured for 24, 48, 72 and 96 h. Subsequently, 10 µl 5 mg/ml MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to the cells for 4 h at 37°C. The medium was gently aspirated and 100 µl dimethyl sulfoxide was added and the cells were incubated for 15 min. Absorbance was measured at a wavelength of 490 nm using a microplate reader. The cells transfected with N.C. shRNA were used as negative controls and the experiment was repeated three times.

Immunohistochemical analysis. Immunohistochemical staining was performed to detect the expression and distribution of HMGB1 in rectal cancer tissues. Briefly, the sections were dewaxed in xylene for 5-10 min and were exposed to a graded alcohol solution at concentrations of 100, 95, 90, 80 and 70%, respectively, for 3-5 min for rehydration. Following this, the
sections were placed in distilled water for 3 min. Finally, the sections were sealed with neutral gum and incubated overnight with a primary anti-HMGB1 antibody at room temperature. The specimens were then washed three times with phosphate-buffered saline for 5 min, and were incubated with goat anti-mouse secondary antibody for 1 h at room temperature. The stained slides were visualized and images were captured using a Nikon Eclipse Ti microscope (Nikon Corporation, Tokyo, Japan). The positivity of immunostaining was identified in eight different fields at 400x magnification.

Western blotting. For total protein, the samples and cells were washed in phosphate-buffered saline and were lysed in radioimmunoprecipitation assay buffer [50 mmol/l of Tris, 1% NP-40, 150 mmol/l of NaCl, 1 mmol of EDTA, 1% sodium dodecyl sulfate (SDS) and 0.25% SDC]. When assessing cytoplasmic or nuclear proteins, a nuclear protein extraction kit (Beyotime Institute of Biotechnology, Beijing, China), according to the manufacturer's protocol. The concentration of whole proteins in the lysates was determined using the Bradford assay (Beyotime Institute of Biotechnology). Protein samples (10 µg) were separated by 10% SDS-PAGE and were then transferred electrophoretically onto a nitrocellulose membrane. Subsequently, the membrane was blocked with 5% bovine serum albumin (Beyotime Institute of Biotechnology) in Tris-buffered saline containing Tween (50 mmol/l Tris-HCl, 150 mmol/l NaCl and 0.1% Tween). The membranes were incubated with primary antibodies overnight at 4°C. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse (cat. no. sc-2031; 1:1,000) or goat anti-rabbit immunoglobulin G (cat. no. sc-2004; 1:1,000; each from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) secondary antibodies for 1 h at room temperature. The resulting bands were detected using an enhanced chemiluminescence western blotting detection system (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. β-actin was used as an internal reference. The gray value of the protein band was analyzed using ImageJ version 1.38 (NIH, Bethesda, MD, USA).

Statistical analysis. Statistical analyses were performed using SPSS 20.0 statistical software (IBM SPSS, Armonk, NY, USA). Clinical indicators were compared using $\chi^2$ test. The data are presented as the mean ± standard deviation. P<0.01 was considered to indicate a statistically significant difference.

Results

**HMGB1 is highly expressed in samples from patients with rectal cancer, as detected by immunohistochemical analysis.** A total of 51 patients and 51 normal controls were studied in the present study; the median age was 60.47 years, with a range of 26-85 years. HMGB1 expression was detected by immunohistochemical analysis, and images of the specimens were captured (original magnification, x400; Fig. 1). HMGB1 protein was predominantly located in the cytoplasm and nucleus of rectal tumor cells, with control tissues exhibiting no positive staining for HMGB1. Positive HMGB1 expression appeared as brown cytoplasmic and nuclear staining. The whole panel of rectal cancer and normal tissues was tested.

![Figure 1](image)

**Figure 1. Immunohistochemical detection of high-mobility group protein 1 (HMGB1) in rectal cancer and normal tissues. Paraffin-embedded samples were analyzed by immunohistochemistry. (A and B) Rectal cancer tissues. HMGB1 protein was mainly located and expressed in the cytoplasm and nucleus of rectal tumor cells. (C and D) Normal tissues, which were used as negative controls. Negative expression of HMGB1 was detected in the control tissues (original magnification, 400x).**

<table>
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<tr>
<th>Group</th>
<th>Normal tissues</th>
<th>Rectal cancer tissues</th>
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<tbody>
<tr>
<td>HMGB1</td>
<td>2 (3.92%)</td>
<td>49 (96.08%)</td>
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<tr>
<td>P-value</td>
<td>&lt;0.001</td>
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**Table I. HMGB1 expression in rectal cancer and normal tissues.**

As presented in Table I, the positive rate of HMGB1 expression was 96.08% (49/51) in rectal cancer tissues, which was significantly higher than in normal tissues (3.92%; 2/51).

**Western blot analysis of HMGB1 expression in rectal cancer tissues.** In order to further detect the expression levels of HMGB1 in rectal cancer, rectal cancer tissues, peritumoral tissues and normal control tissues (three specimens each) underwent western blotting. As shown in Fig. 2A, the expression levels of HMGB1 were markedly increased in rectal cancer tissues compared with in peritumoral or normal control tissues, which was consistent with the immunohistochemical results. β-actin was used as an internal reference. As shown in Fig. 2B, the data were normalized to β-actin and expressed as mean ± standard deviation. The expression levels of HMGB1 were significantly higher in rectal cancer tissues compared with in peritumoral tissues (P<0.01) or normal control tissues (P<0.01).

**HMGB1 is located in the cytoplasm and nucleus of colorectal cancer cells.** Three colorectal cancer cell lines were selected
as a cell model. Western blotting was performed to detect the expression of HMGB1 in Colo320, SW620 and HCT116 cells (Fig. 3). In order to detect the location of HMGB1, cytoplasmic and nuclear proteins were extracted, according to the kit protocol. β-tubulin was used as a cytoplasmic marker and Lamin B1 was used as a nuclear marker. The results demonstrated that HMGB1 was mainly located in the nucleus, but was also partly distributed in the cytoplasm of colorectal cancer cells. These findings are consistent with the results of the immunohistochemical analysis.

**HMGB1 expression is successfully silenced by HMGB1-specific shRNA.** In order to further confirm the role of HMGB1 in cancer cells, RNA interference technology was used to knockdown the expression of HMGB1 in SW620 cells. Three siRNA pairs specific to HMGB1 were generated to interfere with endogenous HMGB1 expression in SW620 cells; however, the knockdown efficiency was not significantly altered compared with that of the N.C. siRNA (Fig. 4A). Therefore, HMGB1-specific shRNA was used to knockdown the endogenous expression of HMGB1 in SW620 cells. As shown in Fig. 4B, transfection with HMGB1-shRNA for 48 h effectively downregulated the endogenous expression of HMGB1, as determined by western blotting.

**Knockdown of endogenous HMGB1 expression inhibits the proliferation of colorectal cancer cells.** The present study aimed to determine whether knockdown of endogenous HMGB1 expression would affect the proliferation of colorectal cancer cells. Therefore, two cell lines were used as a cell model and an MTT assay was conducted to detect the proliferation of cancer cells transfected with HMGB1 shRNA. As shown in Fig. 5, the HMGB1 shRNA-transfected SW620 cells had much lower proliferative ability compared with the untreated or N.C. shRNA-transfected cancer cells. In addition, the survival rate of HMGB1 shRNA-transfected Colo320 cells was determined by MTT assay. Knockdown of endogenous HMGB1 expression inhibited the proliferation of Colo320 cells, compared with those transfected with N.C. shRNA; however, the results demonstrated that SW620 cells were more appropriate for transfection with shRNA.
Knockdown of endogenous HMGB1 expression activates caspase-3 and its substrate poly (ADP-ribose) polymerase (PARP). The present study indicated that knockdown of HMGB1 expression significantly inhibited the proliferation of SW620 and Colo320 cells. Subsequently, the present study aimed to determine whether cell apoptosis was induced in HMGB1 shRNA-transfected cells. Caspase-3 is the executor of cell apoptosis, and the activation of caspase-3 and its substrate PARP were detected using western blotting. As shown in Fig. 6, the expression levels of cleaved caspase-3 were markedly enhanced in HMGB1 shRNA-transfected SW620 cells, compared with in the untreated or N.C. shRNA-transfected cells. Furthermore, the expression levels of PARP were decreased, whereas the levels of cleaved PARP were upregulated in HMGB1 shRNA-transfected SW620 cells. These data suggest that transfection with HMGB1 shRNA may significantly increase cell apoptosis in colorectal cancer cells.

Knockdown of endogenous HMGB1 contributes to the activation of cell apoptosis-associated proteins. The present study also detected the expression levels of the cell apoptosis-associated proteins Bcl-2 and Bax. Bax is an apoptosis inducer, whereas Bcl-2 is an apoptosis inhibitor. The Bax/Bcl-2 ratio reflects the situation of cell apoptosis. In the present study, the expression levels of Bax and Bcl-2 were detected by western blotting, and the results demonstrated that knockdown of HMGB1 resulted in downregulation of Bcl-2 and upregulation of Bax; therefore, the Bax/Bcl-2 ratio was increased in the HMGB1 shRNA-transfected colorectal cancer cells (Fig. 7). These results indicate that knockdown of endogenous HMGB1
expression may activate the intrinsic mitochondrial apoptotic pathway.

Discussion
HMGB1 is a recently discovered oncogene that is associated with tumor progression and metastasis. It has previously been reported that HMGB1, through interactions with RAGE and TLRs, induces inflammation and promotes cancer progression (22). In addition, it has been demonstrated that HMGB1 may induce autophagy or apoptosis in cancer cells, depending on its redox status (23). Flohr et al (10) analyzed the expression patterns of HMGB1 in 13 breast cancer samples, and northern blot analyses of the 1.4 kb and 2.4 kb transcripts of HMGB1 revealed a strong intertumoral variation by a factor of 8.5 and 14.5, respectively, indicating that HMGB1 may be a marker of considerable clinical interest. The role of HMGB1 has been discussed in several types of cancer, including breast cancer (24), colorectal cancer (9,25), gastric cancer (26-28), non-small cell lung cancer (29), pancreatic cancer (30), head and neck cancer (31), and gallbladder cancer (32). However, the role of HMGB1 in rectal cancer remains to be elucidated. The present study collected rectal cancer specimens, and used colorectal cancer cell lines as cell models to explore the role of HMGB1 in the progression of rectal cancer. The aim of the present study was to identify novel targets in rectal cancer therapy.

In the present study, immunohistochemistry and western blotting were used to analyze the expression levels of HMGB1 in pathological specimens from patients with clinically identified rectal cancer. In addition, the distribution of HMGB1 in tumor cells was detected by western blot analysis. The results demonstrated that HMGB1 was highly expressed in specimens from patients with rectal cancer. Notably, HMGB1 was distributed and located not only in the nucleus, but also in the cytoplasm of colorectal cancer cells. These results indicated that HMGB1 may have an important role in the progression and development of rectal cancer. Consistent with these findings, Song et al (18) demonstrated that HMGB1 was overexpressed in ~85% of gastric cancers, and downregulation of HMGB1 increased the proportion of cells in G0/G1, phase and sensitized gastric cancer cells to apoptosis via the caspase-3 pathway.

Livesey et al (33) hypothesized that direct molecular interactions between HMGB1 and TP53 in colorectal cancer regulated the balance between apoptosis and autophagy through regulation of the cytosolic localization of the reciprocal binding partner. Increased cytosolic HMGB1 was shown to enhance autophagy, whereas increased cytosolic TP53 enhanced apoptosis in colon cancer cells. In addition, Zhang et al (21) reported that knockdown of HMGB1 inhibited growth and invasion of gastric cancer cells via the nuclear factor-xB pathway in vitro and in vivo.

In conclusion, the present study demonstrated that knockdown of endogenous HMGB1 expression in SW620 and Colo320 colorectal cancer cells was able to significantly inhibit cell proliferation. In addition, apoptosis was induced in HMGB1 shRNA-transfected colorectal cancer cells. However, the mechanism underlying the effects of HMGB1 on the regulation of cell apoptosis requires further study, and the downstream targets or receptors of HMGB1 remain to be clearly explored.

References


