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*Original Article*

# Effects of siRNA interference and over-expression of HMGA2 on proliferation and apoptosis of colorectal cancer cells

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**Abstract:** High mobility group A2 (HMGA2) is one oncogenic gene that can regulates cell proliferation, cycle and apoptosis. Up-regulation of HMGA2 is correlated with multiple tumors. This study constructed interference and over-expression vector targeting HMGA2, to investigate if HMGA2 plays a role in regulating proliferation or apoptosis of colorectal cancer cells. Colorectal cancer patients admitted in our hospital were collected for tumor and adjacent tissue samples. qRT-PCR and immunofluorescence assay were used for testing HMGA2 expression. HCT116 and NCM460 cells were compared to expression of HMGA2 and cyclin A. Flow cytometry was used to detect cell proliferation, cycle and apoptosis. *In vitro* cultured HCT116 cells were treated with si-HMGA2 or pIRES2-HMGA2, followed by qRT-PCR, Western blot and flow cytometry for expression of HMGA2 and cyclin A, and cell proliferation, cycle or apoptosis. Compared to adjacent tissues, colorectal cancer tissues had significantly elevated HMGA2 mRNA/protein expression. HCT116 cell had significantly elevated expression of HMGA2/cyclin A, S phase or G2/M phase ratio, and proliferation potency than those of NCM460 cells, whilst G0/G1 ratio and apoptotic rate were lower. Transfection of HMGA2 siRNA remarkably decreased cyclin A expression in HCT116 cells, weakened its proliferation potency, elevated G0/G1 ratio whilst decreased S phase or G2/M phase ratio, and induced apoptosis. Over-expression of HMGA2 exerted opposite effects. Up-regulation of HMGA2 occurs in colorectal cancer tissues. HMGA2 can facilitate proliferation and colorectal cancer cells and inhibit apoptosis via up-regulating cyclin A expression, accelerating G1/S or G2/M phase transition.

**Keywords:** HMGA2, cyclin A, colorectal cancer, cell proliferation, cell cycle, apoptosis

## Introduction

Colorectal cancer (CRC) is one common malignant tumor in digestive tract and as higher incidence, as it is the third popular tumor in gastrointestinal system, frequently occurring at 40~50 years people [1]. With transition of life styles in China, the occurrence rate of CRC is gradually increasing, severely threatening public health [2]. High mobility group A2 (HMGA2) is one member of HMGA family and is widely distributed in eukaryotes. It is named after the rapid migration feature during polyacrylamide gel electrophoresis (PAGE) [3]. As one non-histone chromosomal protein, HMGA2 itself has no transcriptional function but can regulate such functions [4]. During early phase of embryonic development and immature tissues, HMGA2 has sufficient expression, which is gradually decreased with tissue/organ differentiation. In various tumor tissues, however, HMGA2 is abundantly expressed [3]. HMGA2 is widely involved in the regulation of cell proliferation [5], cell cycle and apoptosis [6], migration [7] and differentiation via multiple mechanisms. Abnormally elevated expression of HMGA2 is correlated with occurrence of multiple tumors such as prostate cancer [8], gall bladder cancer [7], gastric carcinoma [9], pulmonary carcinoma [10] and ovarian cancer [11], and affects tumor progression, metastasis and recurrence. This study observed the effect of HMGA2 interference or over-expression on proliferation, cell cycle and apoptosis of CRC cells, in order to investigate possible role of HMGA2 in CRC pathogenesis.

## Materials and methods

### *Major reagents and materials*

Human CRC cell line HCT116 and normal colon epithelial cells NCM460 were purchased from Hongshun Biotech (China). RPMI1640 medium, DMEM medium, FBS and penicillin-streptomycin were purchased from Gibco (US). Trizol and Lipofectamine 2000 were purchased from Invitrogen (US). ReverTra Ace qPCR RT Kit and SYBR Green dye were purchased from Toyobo (Japan). HMGA2 siRNA was synthesized by Gimma Bio (China). Mouse anti-HMGA2 antibody and rabbit anti-cyclin A antibody were purchased from Abcam (US). Goat anti-mouse IgG Alexa Fluor 488 fluorescent secondary antibody and horseradish peroxidase (HRP) labelled secondary antibody were all purchased from ImmunoResearch (US). Annexin V/PI apoptotic kit was purchased from Youningwei Bio (China). CFSE cell proliferation and tracing kit was purchased from Beyotime (China).

### *Clinical information*

A total of 30 CRC patients (19 males and 11 females, aging between 43 and 71 years, average age = 57.6 years) who received treatment in Xianning Central Hospital from November 2015 to May 2016 were recruited in this study. Tumor tissues and adjacent tissues were collected for post-op pathology examination. All patients had not received chemo/radio-therapy or specific anti-tumor treatment before surgery. All patients have signed informed consents regarding this study, which has been reviewed by the ethical committee of Xianning Central Hospital.

### *Immunofluorescence assay for HMGA2 expression in tumor tissues*

Both CRC and adjacent tissues collected from the surgery were fixed in 10% formalin, and dehydrated in 20% sucrose. Serial cryosection (8 μm) was prepared and dried at room temperature for 30 min. After rinsing in PBS for three times (5 min each), normal goat serum was used for 60 min blocking. Mouse anti-human HMGA2 primary antibody (1:200 dilution) was added for 4°C overnight incubation. On the next day, PBS was used to rinse tissues for three times, followed by incubation at fluorescent secondary antibody (1:400 dilution), and PBS washing (5 min, 3 times). DAPI was used to stain tissues, followed by PBS washing and mounting coverslips by neutral resin. A fluorescent microscope was used for capturing images for analysis.

### *Cell culture*

HCT116 and NCM460 cells were cultured in RPMI1640 complete medium in a 37°C chamber with 5% $CO_2$. Culture medium was changed every 3 days and cells were passed based on growth conditions. During each passage, original medium was removed, followed by PBS washing. Cells were digested in 0.6 mL trypsin until shrinkage and oval shape with partial detachment. 2 mL complete medium was added for re-suspend cells, which were inoculated in other culture dishes for further assay.

### *Construction of HMGA2 over-expression plasmid*

CDS fragment of HMGA2 gene was amplified. Targeted sequence was determined by gel electrophoresis and was re-cycled from gel extraction. DNA fragment was digested by XhoI and BamHI enzymes and was ligated to pIRES2 plasmids. Recombinant plasmid was then used to transfect JM109 competent cells, which were screened on ampicillin-containing culture plate. Positive bacterial clones were further amplified to extract recombinant plasmids containing target fragments. Sequencing was then performed to determine the correct insertion of plasmid, which was named as pIRES2-HMGA2. Empty plasmid pIRES2-Blank was used as the control.

### *Experimental grouping and cell transfection*

*In vitro* cultured HCT116 cells were divided into four groups: si-NC, si-HMGA2, pIRES2-Blank and pIRES2-HMGA2 groups. Sequences were: si-HMGA2F, 5'-CAGCC UGAAU AACUU GAACT T; si-HMGA2R, 5'-GUUCA AGUUA UUCAG GCUGT T-3'; si-NCF, 5'-UUCUC CGAAC GUGUC ACGUT T-3'; si-NCF, 5'-ACGUG ACACG UUCGG AGAAT T-3'. Lipofectamine 2000 was used for transfection following manual instruction. Cells were collected for test 72 h later.

### *qRT-PCR for gene expression*

Trizol method was used to extract total RNA, whose concentration was quantified by Eppendorf nucleic acid/protein analyzer. ReverTra Ace qPCR RT Kit was used for synthesize cDNA us-

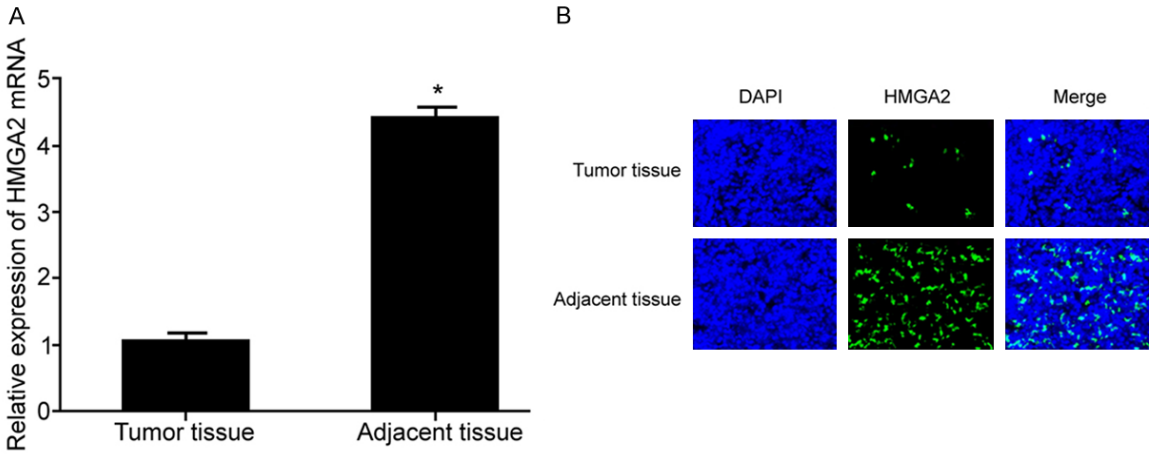


**Figure 1.** Elevated HMGA2 expression in CRC tissues. A. qRT-PCR for gene expression; B. Immunofluorescence for HMGA2 protein expression. *, P<0.05 compared to adjacent tissues.

ing RNA as the template by reverse transcription. cDNA products were stored at -20°C for further use. Using β-actin as the internal reference gene, PCR amplification was performed on Bio-Rad CFX96 fluorescent quantitative PCR cycler using cDNA as the template. mRNA relative expression level was calculated by $2^{-\Delta\Delta Ct}$ method. Primers used were: HMGA2 $P_F$: 5'-ACCCA GGGGA AGACC CAAA-3'; HMGA2 $P_R$: 5'-CCTCT TGGCC GTTTT TCTCC A-3'; Cyclin A $P_F$: 5'-ACATG GATGA ACTAG AGCAG GG-3'; Cyclin A $P_R$: 5'-GAGTG TGCCG GTGTC TACTT-3'; β-actin $P_F$: 5'-GAACC CTAAG GCCAA C-3'; β-actin $P_R$: 5'-TGTCA CGCAC GATTT CC-3'.

*Western blot*

RIPA cell lysis buffer was used to extract proteins, whose concentration was determined by BCA protein quantification kit. 40 μg protein was separated by 10% SDS-PAGE and was separated onto PVDF membrane, which was blocked in 5% defatted milk powder and incubated in primary antibody (1:200 dilution for HMGA2, 1:200 dilution for Cyclin A, and 1:500 dilution for β-actin) for 4°C overnight incubation. After PBST rinsing, HRP labelled secondary antibody (1:5,000 dilution) was added for 37°C incubation for 60 min. ECL reagent was used for develop the membrane. After scanning, images were kept for analysis using β-actin as the internal reference.

*Flow cytometry for cell cycle*

Trypsin was used to digest cells, which were rinsed in cold PBS and were fixed in cold 70% ethanol for 24 h. After PBS washing, PI staining dye was added for 37°C overnight incubation for 30 min. A total of 10,000 cells were counted for flow cytometry assay.

*CFSE staining for cell proliferation*

Single cell suspension was prepared after trypsin digestion, with the addition of equal volume of CFSE working solution. Cells were cultured at 37°C chamber for 10 min, and were treated with 40% volume of cold bovine serum for 10 min to stop staining. Complete culture medium was centrifuged and rinsed twice. Cells were re-suspended in culture medium. Cells after tagging were seeded into 6-well plate for measuring fluorescent strength after 48 h incubation.

*Flow cytometry for cell apoptosis*

Cells were digested by trypsin and were collected. After rinsed in cold PBS twice, 500 μL 1× Binding Buffer and 5 μL Annexin V-FITC were mixed for dark incubation for 15 min in dark at room temperature. 5 μL PI was added for 1 h incubation, followed by loading and test.

*Statistical analysis*

SPSS 18.0 was used for data input and statistical analysis. Measurement data were presented as mean ± standard deviation (SD), and were compared by two independent sample t-test. A statistical significance was defined when P<0.05.

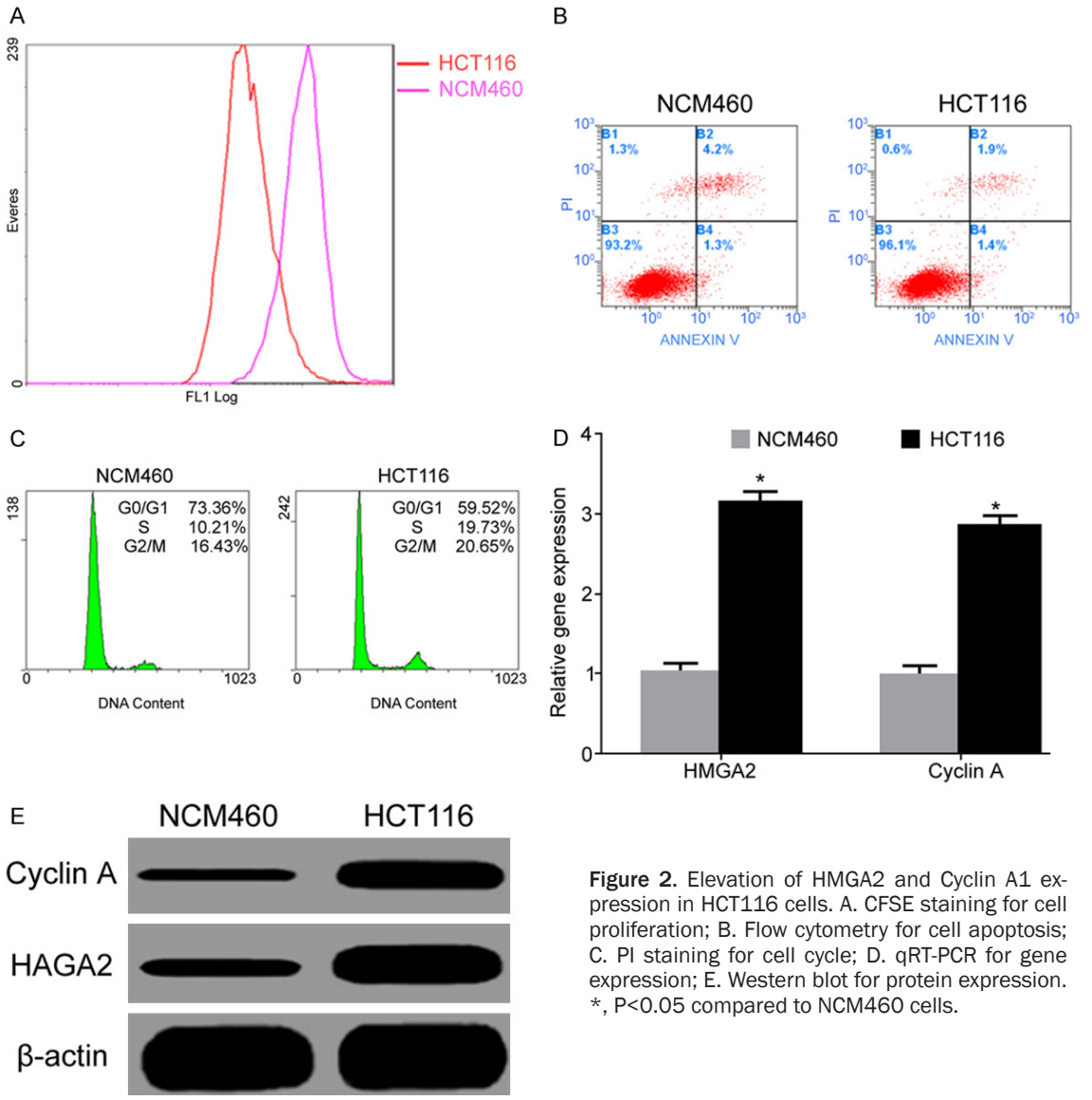


**Figure 2.** Elevation of HMGA2 and Cyclin A1 expression in HCT116 cells. A. CFSE staining for cell proliferation; B. Flow cytometry for cell apoptosis; C. PI staining for cell cycle; D. qRT-PCR for gene expression; E. Western blot for protein expression. *, P<0.05 compared to NCM460 cells.

## Results

*Elevated HMGA2 expression*

qRT-PCR results showed significantly elevated HMGA2 mRNA expression level in CRC tumor tissues compared to adjacent tissues (**Figure 1A**). Immunofluorescence assay revealed significantly higher HMGA2 protein expression than adjacent tissues (**Figure 1B**).

*Elevated HMGA2 and cyclin A1 expression in HCT116 cells*

CFSE staining results showed significantly higher proliferation ability of HCT116 cells than NCM460 cells (**Figure 2A**). Flow cytometry results also revealed lower apoptotic rate in HCT116 cells than NCM460 (**Figure 2B**). A significant difference also existed in cell cycle (**Figure 2C**). qRT-PCR results showed remarkably higher mRNA/protein level of HMGA2 and Cyclin A in HCT116 cells compared to NCM460 cells (**Figure 2D** and **2E**).

*Interference and over-expression of HMGA2 inhibited or facilitate HCT116 cell proliferation*

The transfection of HMGA2 siRNA and over-expression plasmid significantly depressed HMGA2 and Cyclin A mRNA (**Figure 3A**) or protein (**Figure 3B**) expression in HCT116 cells. After down-regulation of HMGA2, proliferation ability of HCT116 cells was significantly weakened

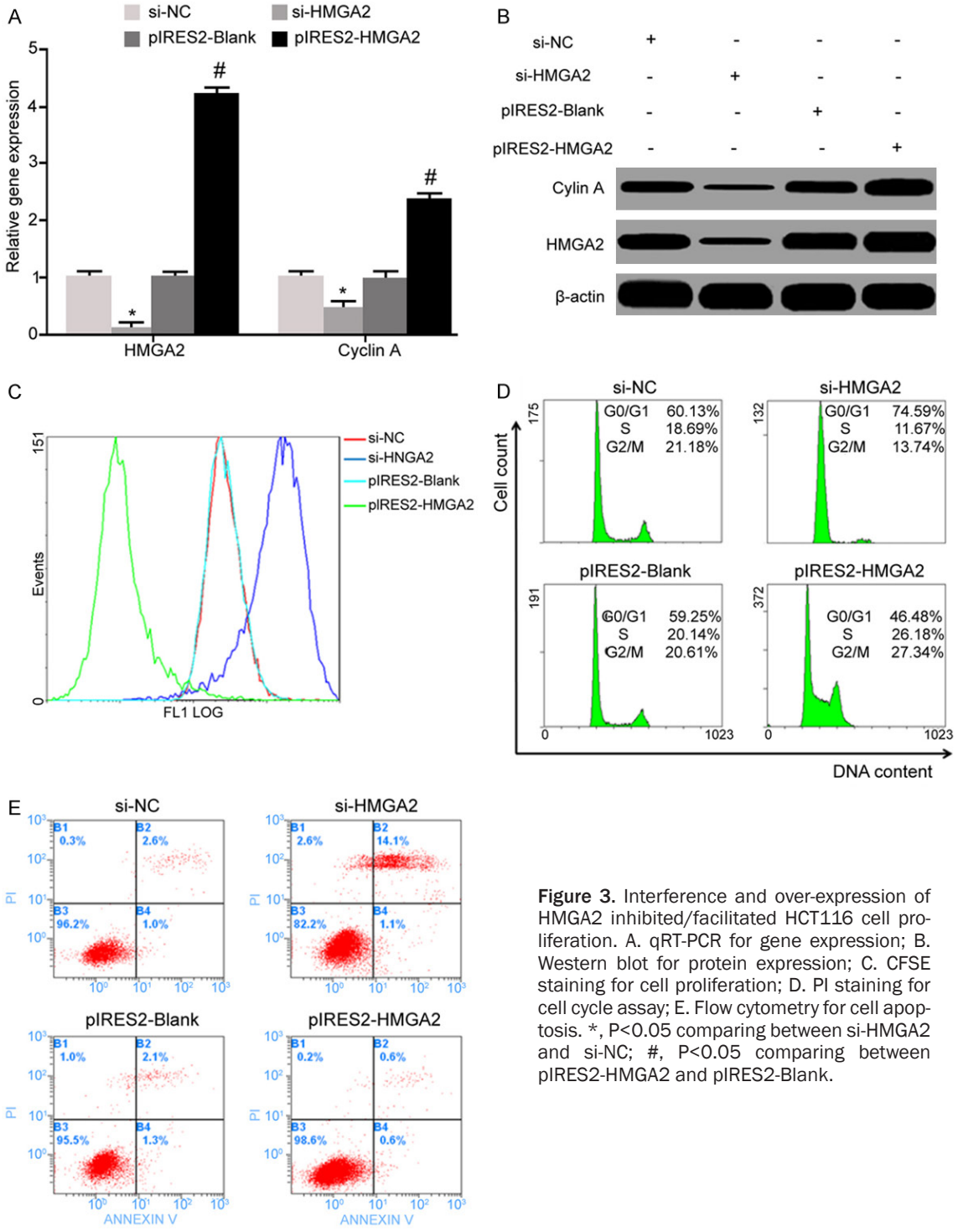


**Figure 3.** Interference and over-expression of HMGA2 inhibited/facilitated HCT116 cell proliferation. A. qRT-PCR for gene expression; B. Western blot for protein expression; C. CFSE staining for cell proliferation; D. PI staining for cell cycle assay; E. Flow cytometry for cell apoptosis. *, P<0.05 comparing between si-HMGA2 and si-NC; #, P<0.05 comparing between pIRES2-HMGA2 and pIRES2-Blank.

(**Figure 3C**). Meanwhile, G0/G1 phase ratio was increased whilst S phase and G2/M phase ratio were decreased (**Figure 3D**) to induce more apoptosis (**Figure 3E**). Over-expression of HMGA2 had opposite effects as shown in **Figure 3C-E**.

## Discussions

CRC is one common malignant tumor worldwide, with relatively high incidence and mortality, plus lower 5-year survival rate [12]. Therefore, detailed research of molecular mecha-

nism underlying CRC onset and pathogenesis, search for more effective molecular target for treating CRC, exploration of new methods to decrease morality and to improve prognosis, are still major challenges in CRC related research.

HMGA2 protein is one member of HMGA family (including four members, HMGA1a, HMGA1b, HMGA1c and HMGA2) [3]. The gene encoding human HMGA2 locates on q15 of chromosome 12, and is composed of 5 exons and 4 introns [13]. HMGA2 protein is one non-histone chromosome protein without transcriptional function itself, but can change chromatin conformation, thus affecting nuclear biological processes including gene transcription, replication, rearrangement and repair of DNA injury, and participating embryonic development, tissue/organ formation and tumor pathogenesis [14].

HMGA2 participates in the regulation of cell proliferation, cell cycle and apoptosis via multiple mechanisms. Previous studies showed abnormally high expression of HMGA2 in prostate cancer [8], bladder cancer [7], gastric carcinoma [9], pulmonary cancer [10] and ovarian cancer [11]. This study observed the effect of interference or over-expression of HMGA2 on proliferation, cycle and apoptosis of CRC cells, thus investigating possible role of HMGA2 in CRC pathogenesis. Our results showed significantly elevated HMGA2 expression in CRC tumor tissues compared to adjacent tissues. Fan et al showed abnormally elevated HMGA2 expression in CRC patient tumor tissues [15]. Liu et al revealed remarkably elevated HMGA2 expression in tumor tissue and serum of CRC patients, whose overall survival rate, disease progression-free survival rate were lower for those patients with higher HMGA2 expression [16]. Razzi et al proposed significantly elevated expression of HMGA2 in CRC tumor tissues, and close correlation between vascular metastasis and the expression level of HMGA2, which thus can work as one index predicting tumor progression and clinical endpoints [17]. Yu et al showed positive correlation between HMGA2 expression and clinical stage of CRC patients [18]. Wang et al revealed 3.53-fold higher metastatic rate in those with higher HMGA2 expression level than those with lower HMGA2 (95% CI: 1.35~9.70) [19], plus significantly worse prognosis. During this study, HMGA2 expression was found to be potentiated in CRC tumor tissues, as consistent with Fan et al [15], Liu et al [16] and Razzi et al [17].

Cell cycle protein A (Cyclin A) can interact with CDK1 and CDK2, and plays a crucial role in accelerating G1/S phase transition, G2/M phase transition, facilitating cell mitosis and proliferation [20]. Tessari et al found the HMGA2 could interact with p120E4F and potentiate intracellular expression of Cyclin A, to accelerate cell cycle progression and mitotic proliferation, thus being involved in tumor occurrence [21]. This study revealed higher expression of HMGA2 and Cyclin A1, plus ratios of S phase and G2/M phase in HCT116 cells compared to NCM460 cells, indicating that HMGA2 might play a role in up-regulating Cyclin A expression, and facilitating proliferation of CRC cells. Furhter assay showed that transfection of HMGA2-siRNA remarkably decreased Cyclin A expression in HCT116 cells and decreased the proliferation activity, increased G0/G1 phase ratio whilst decreased S phase or G2/M phase ratio, and induced cell apoptosis. Transfection of HMGA2 over-expression plasmid resulted in opposite effects. Kao et al showed the correlation between HMGA2 and proliferation, epithelial-mesenchymal transition (EMT), and migration regulation of CRC [22]. After silencing HMGA2 expression using siRNA, proliferation or survival of CRC cells was significantly decreased, with potentiated sensitivity for chemo-drugs. Siahmansouri et al [23] found that siRNA application significantly induced death of CRC cell HT-29, decreased MMP-9 expression and inhibited invasion of CRC. Wu et al showed that HMGA2 down-regulation significantly inhibited proliferation potency of CRC cell HCT-116 and SW480 [24]. Over-expression of HMGA2 remarkably potentiated its activation effect on PI3K/AKT signal pathway, and enhanced drug resistance against 5-fluouracil. Esmailzadeh et al showed that siRNA interference on HMGA2 significantly induced apoptosis of CRC HCT116 cell apoptosis and G2/M phase arrest [25]. Wang et al found that HMGA2 over-expression weakened clearance potency of γ-H2AX in HCT-116 or SW480 cells after γ-irradiation [19], indicating that HMGA2 over-expression decreased DNA injury repair function of CRC cells. All these abovementioned studies showed pluripotent function of HMGA2 on CRC cells, including proliferation, EMT, cell invasion, DNA repair post-injury, and drug resistance. This study revealed that HM-

GA2 could affect proliferation, cycle and apoptosis of CRC cells via up-regulating Cyclin A expression, which has not been reported previously regarding the correlation between HMGA2 and CRC pathogenesis.

## Conclusion

In tumor tissues of CRC patients, HMGA2 expression level is significantly increased. HMGA2 can accelerate G1/S phase and G2/M phase transition via up-regulating Cyclin A expression, thus facilitating proliferation of CRC tumor cells whilst inhibiting their apoptosis.

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## Disclosure of conflict of interest

None.

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