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Original Article

High mobility group box 3 promotes cervical cancer proliferation by regulating Wnt/β-catenin pathway

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Conflict of Interest
No potential conflict of interest relevant to this article was reported.

ABSTRACT

Objective: High mobility group box 3 (HMGB3) plays an important role in the development of various cancer. This study aims to explore whether HMGB3 regulates cervical cancer (CC) progression and elucidate the underlying mechanism.
Methods: HMGB3 expression in clinical patients' tumor samples were determined by real-time quantitative polymerase chain reaction (qRT-PCR) and western blot. HMGB3 overexpression/knockdown were used to investigate its function. Cell apoptosis and cycle were detected by Annexin V/PI staining and flow cytometry. In vivo tumor model was made by subcutaneous injection of HeLa cells transfected with shRNAs targeting HMGB3 (sh-HMGB31) into the flank area of nude mice. Western blot was used to detect the levels of β-catenin, c-Myc, and matrix metalloproteinase-7 (MMP-7) in Hela and CaSki cells transfected with sh-HMGB3 or shRNAs targeting β-catenin.
Results: Both messenger RNA and protein levels of HMGB3 were upregulated in CC tissues from patients. High expression level of HMGB3 had positive correlation with serosal invasion, lymph metastasis, and tumor sizes in CC patient. Functional experiments showed that HMGB3 could promote CC cell proliferation both in vitro and in vivo. The expression levels of c-Myc and MMP-7 were increased, resulting in regulating cell apoptosis, cell cycle, and activating Wnt/β-catenin pathway.
Conclusions: Our data indicated that HMGB3 may serve as an oncoprotein. It could be used as a potential prognostic marker and represent a promising therapeutic strategy for CC treatment.

Keywords: HMGB3 Protein; Cell Proliferation; beta Catenin; Cervical Cancer; shRNA

INTRODUCTION

Cervical cancer (CC), the third most common gynecological malignancy worldwide, has been estimated to cause 530,000 deaths each year [1,2]. There are various therapies such as surgery, chemotherapy, and radiation therapy in the treatment for CC, but the prognosis is still poor. In developing countries, CC is still the leading cause of deaths from cancers in women [3]. The main reason of poor prognosis in patients with CC is distant metastasis and lymph node metastasis. Early screening technology has led to reduced mortality in CC patients, but even in early CC, tumor metastasis leads to negative effect and results in

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Author Contributions

Conceptualization: Z.S., D.Y.; Data curation: D.Y.; Formal analysis: Y.X.; Investigation: Y.X.; Methodology: L.M., L.Y.; Project administration: L.M.; Resources: Z.S., L.Y., D.N.; Software: D.N.; Writing - original draft: Z.S., D.Y.; Writing - review & editing: D.Y.

about 40% of 5-year survival rate [4,5]. Therefore, it is important to exploit the underlying biological mechanisms of the disease. It will help find novel biomarkers for diagnosis and improve CC treatment strategies.

High mobility group box 3 (HMGB3), along with HMGB1, HMGB2 and HMGB4, belongs to the subfamily of high mobility group protein [6]. It is a kind of non-histone chromosome-binding proteins that are widely present in conserved eukaryotic cells. They exert vital functions in not only DNA replication and repair, but also transcription and recombination [7-9]. HMGB3 is also involved in maintaining the self-renewal and differentiation status of murine hematopoietic stem cells [6]. Recent studies have shown that the gene encoding HMGB3 protein is a new tumorigenic gene in the pathogenesis of leukemia [10]. Overexpression of HMGB3 is positively correlated with poor leukemia prognosis caused by unconventional self-renewing progenitor-like cancer stem cells [11]. HMGB3 in peripheral blood has been identified as a biological indicator of lung cancer. It is also found highly expressed in patients with acute lymphoblastic relapse, gastric adenocarcinoma, lung cancer, and advanced breast cancer [12-15]. Furthermore, HMGB3 RNA interference inhibits the growth of gastric cancer cells [16]. However, the function of HMGB3 in human CC is still unclear.

Wnt/β-catenin signaling pathway plays vital roles in regulating many biological processes including differentiation, proliferation, and apoptosis [17]. Activation of Wnt/β-catenin signaling has been shown to promote the development of a variety of cancers like lung, colorectal, and liver cancer [17]. Recently, studies have also shown that high expression level of β-catenin is observed in CC progression [17] and is considered to be a poor prognostic factor for CC [18]. Taken together, these studies indicate that Wnt/β-catenin signaling is involved in CC development. Therefore, Wnt/β-catenin signaling may be a potential target for cancer treatment.

Recent research showed that HMGB3 silencing inhibited non-small cell lung cancer development through regulating Wnt/β-catenin pathway [19]. Another study reported that long noncoding RNA SOX2-OT activated the Wnt/β-catenin signaling pathway through increasing HMGB3 expression and SOX2-OT knockdown, inhibited proliferation and metastasis of prostate cancer cells through modulating miR-452-5p/HMGB3 Axis and inactivating Wnt/β-catenin pathway [20]. In view of the above findings, we speculated that HMGB3 might play an important effect in growth and migration of CC cells via regulation of Wnt/β-catenin pathway. Therefore, we first detected the expression level of HMGB3 in the tumor tissue from CC patients by RT-PCR and western blot to find the correlation between HMGB3 expression and tumor progression. Next, we exploited the functions and potential molecular mechanisms of HMGB3 in CC growth and migration.

MATERIALS AND METHODS

1. Clinical samples

The clinical samples of 58 CC tissues and adjacent non-tumor tissues were collected from the CC patients at the ZIBO Central Hospital. Thirty-six patients were diagnosed with squamous cell carcinoma, while the other 22 patients were adenocarcinoma. These patients had no prior chemotherapy or radiation therapy before surgery. All samples were confirmed by a certified pathologist and staged according to International Federation of Gynecology and Obstetrics staging system for CC. All patients signed written informed consent. The study

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Table 1. HMGB3 expression and clinicopathological factors in cervical cancer patients (n=58)

Variable	HMGB3 mRNA		p-value*
	Low expression (n=26)	High expression (n=32)	
Age (yr)			1.000
<45	10	13	
≥45	16	19	
Tumor size (maximum diameters)			**0.008**
<4 cm	17	9	
≥4 cm	9	23	
Histology			0.594
Squamous	15	21	
Adenocarcinoma	11	11	
FIGO stage			**0.033**
Ib–IIa	15	9	
IIb–IIIa	11	23	
Lymph node metastasis			**0.033**
No	16	10	
Yes	10	22	
Depth of cervical invasion			**0.032**
<2/3	14	8	
≥2/3	12	24	

Bold numbers mean p-values less than 0.05.
FIGO, International Federation of Gynaecology and Obstetrics; HMGB3, high mobility group box 3; mRNA, messenger RNA.
*χ^2 test.

was approved by the Ethics commitment of ZIBO Central Hospital. All procedures performed in the study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The clinicopathological characteristics were listed in **Table 1**.

2. Cell transfection and proliferation assay

CC cell lines of HeLa, CaSki, SiHa, C33A and normal human cervical epithelial cell line (H8), were purchased from ATCC. Cells were maintained in Dulbecco's Modified Eagle's Medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), penicillin (100 U/mL) and streptomycin (100 U/mL) at 37°C in a humidified incubator of 5% CO_2. Lentiviral vectors overexpressing HMGB3 and plasmids of shRNAs targeting HMGB3 (sh-HMGB3), β-catenin (sh-β-catenin) and their negative control shRNAs (sh-NC) were synthesized or constructed by Genechem (Shanghai, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for plasmids transfection. Cell viabilities were detected by Cell Counting Kit-8 (CCK-8; Abcam, Cambridge, UK) at different time points.

The nucleotide sequences of sh-HMGB3: HMGB3 sense: 5′-GACUAUAAGUCGAAAGGAATT-3′ and HMGB3 antisense 5′-UUCCUUUCGACUUAUAGUCTT-3′.

The nucleotide sequences of sh-β-catenin: 5′-CACCGCAGCTGGAATTCTTTCTAACTTCAA GAGAGTTAGAAAGAATTCCAGCTGCTTTTTTG-3′; 5′-GATCCAAAAAAGCAGCTGGAAT TCTTTCTAACTCTCTTGAAGTTAGAAAGAATTCCAGCTGC-3′.

3. RNA extraction and real-time quantitative polymerase chain reaction (qRT-PCR)

The total RNAs were extracted from cells and tissues by TRIzol (Invitrogen) and then transcribed to cDNA using Reverse Transcription Kit (Thermo Fisher Scientific, Shanghai,

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China). qRT-PCR was used to determine HMGB3 messenger RNA (mRNA) expression and normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The results were expressed as fold changes in tumor tissue relative to matched adjacent normal tissue. GAPDH was used as an internal control. Primers were listed as following:

Forward primer for HMGB3: 5′-ATTCGGAATTCCGTATCTGGCCTTTTGAC-3′;
Reverse primer for HMGB3: 5′-CGGTTACTCGGCTTACGCTTGGACTG-3′;
Forward primer for GAPDH: 5′-GACTCATGACCACAGTCCATGC-3′;
Reverse primer for GAPDH: 5′- AGAGGCAGGGATGATGTTCTG -3′;
Forward primer for Ki67: 5′-ACGCCTGGTTACTATCAAAAGG-3′;
Reverse primer for Ki67: 5′-CAGACCCATTTACTTGTGTTGGA-3′.
Forward primer for PCNA: 5′-CCTGCTGGGATATTAGCTCCA-3′;
Reverse primer for PCNA: 5′-CAGCGGTAGGTGTCGAAGC-3′.

4. Flow cytometry

Cell Samples were harvested 24 hours after transfection of vectors or plasmids. Apoptosis detection kit (BD Biosciences, San Jose, CA, USA) was used to detect cell apoptosis by stained with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide (PI). For cell cycle analysis, samples were stained by PI alone using a cell cycle detection kit (BD Biosciences). Apoptosis and cell population rates at G0±G1, S, and G2±M phases were analyzed by flow cytometry.

5. Western blot

Cell or tissue samples were lysed with RIPA lysis buffer supplemented with 1% PMSF (Sigma, St. Louis, MO, USA) to extract total protein. The samples equal to 20 μg protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene fluoride membranes. The membranes were incubated with primary antibodies against HMGB3 (1:500; Cat. No. AF5507; R&D Systems, Inc., Minneapolis, MN, USA), cyclin D3 (1:1,000; ab183338; Abcam), Cdk2 (1:1000; ab32147: Abcam), β-catenin (1:500; Cat. No. 9562S; Cell Signaling Technology, Danvers, MA, USA), c-myc (1:500; Cat. No. sc-373712; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), matrix metalloproteinase-7 (MMP-7, 1:500; Cat. No. sc-80205; Santa Cruz Biotechnology, Inc.), and GAPDH (1:1,000; Cat. No. AF0006; Beyotime Institute of Biotechnology, Haimen, China) at 4°C overnight. After washing the membrane three times, corresponding horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:2,000; Cat. No. sc-2004; Santa Cruz Biotechnology, Inc.) were added and incubated at room temperature for one hour. The electrochemical luminescence (ECL) reagent was put on to observe the protein bands. GAPDH was used as an internal control. Protein expression levels were normalized to GAPDH and quantified by Image J software.

6. Xenograft mouse model

Male BALB/c athymic nude mice of 4 weeks old were kept in a pathogen-free room with a light / dark (12:00/20:00) cycle. They were free access to food and water. One hundred microliter (1×10^7 cells) of Hela cells expressing negative control sh-RNA or sh-HMGB3 were injected subcutaneously on either side of the flank area of nude mice for tumor xenograft. The growth of tumor was examined every 3 days and calculated the tumor volume as length×width^2×0.5. The mice were sacrificed at day 24th after injection and the tumors were excised for subsequent experiments. Animal experiments have been approved by the Institutional Committee for Animal Research of ZIBO Central Hospital and followed+national guidelines for the care and use of laboratory animals.

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7. Statistical analysis

Data were shown as the mean±standard deviation and analyzed by SPSS 20.0 software (IBM Corp., Armonk, NY, USA). Relationships between HMGB3 expression and clinicopathologic characteristics were assessed by χ^2 test. The statistical significance of differences among groups was tested by a 2-tailed Student's t-test or analysis of variance analysis and a post hoc test. $p<0.05$ was considered as statistically significant. All the experiments have been repeated at least 3 times to confirm the results.

RESULTS

1. HMGB3 was overexpressed in CC tissues

We first analyzed the expression of HMGB3 mRNA and protein in 58 CC tissues and adjacent non-tumor tissues to investigate whether HMGB3 was involved in carcinogenesis. The expression level of HMGB3 mRNA in tumor tissues was significantly higher than that in corresponding non-tumor tissues (**Fig. 1A**; $p<0.001$). In addition, according to western blot results, the expression of HMGB3 protein in CC tissues was significantly higher than in adjacent tissues (**Fig. 1B**; $p<0.001$). Moreover, we explored the relationship between clinicopathological factors and HMGB3 expression. The results showed that HMGB3 mRNA expression was significantly higher in patients with larger tumors (≥4 cm) (**Fig. 1C**; $p<0.001$) and patients with advanced International Federation of Gynaecology and Obstetrics (FIGO) stage (**Fig. 1D**; $p<0.001$). These findings indicated that HMGB3 was related to carcinogenesis of CC.

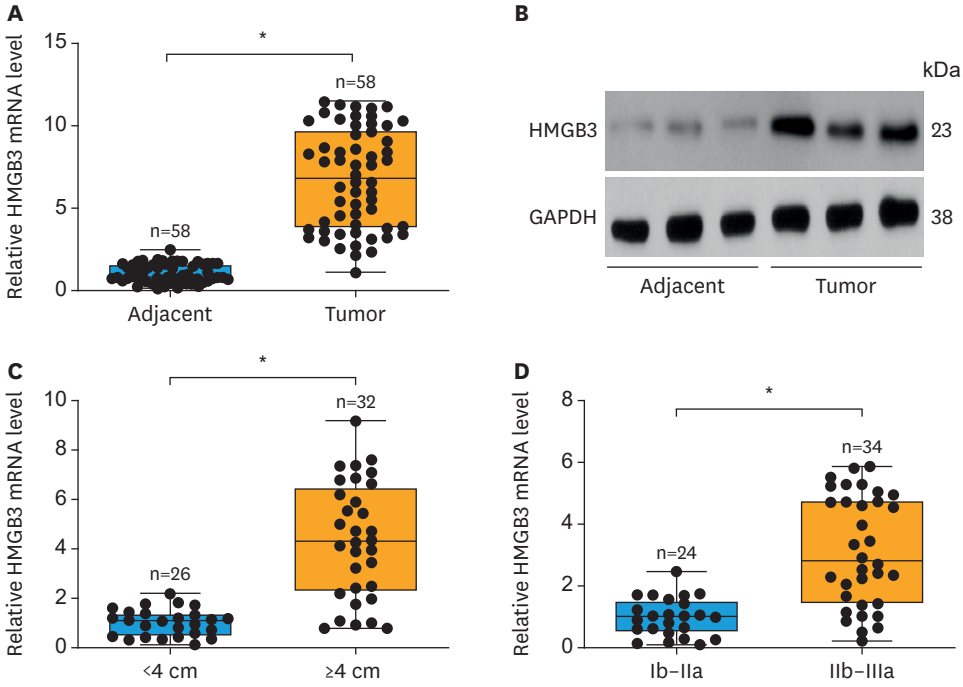

Fig. 1. HMGB3 is overexpressed in cervical cancer tissues. (A) HMGB3 mRNA expression was upregulated in cervical cancer tissues. HMGB3 expression was measured by qRT-PCR and normalized to adjacent tissues (n=58 for each group). (B) Representative western blots of HMGB3 protein expressions between cervical cancer tissues and adjacent tissues. Moreover, HMGB3 expression was significantly higher in patients with bigger tumor size (C) (n=26 for <4 cm and n=32 for ≥4 cm) and advanced FIGO stage (D) (n=24 for <Ib–IIa and n=34 for ≥IIb–IIIa). Data were presented as box plot with all data points. t-tests followed by Mann-Whitney test.
FIGO, International Federation of Gynaecology and Obstetrics; HMGB3, high mobility group box 3; mRNA, messenger RNA; qRT-PCR, real-time quantitative polymerase chain reaction.
*$p<0.001$ between the indicated groups.

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2. Analysis of the relationship between HMGB3 mRNA expression and clinicopathological characteristics in patients with CC

In order to further study the correlation between HMGB3 mRNA and various clinicopathological features in CC patients, the median value of HMGB3 mRNA in all CC tissues was used as the cutoff value, and all patients were divided into high expression groups (n=32) And low expression groups (n=26). Then, the correlation between HMGB3 mRNA expression and clinicopathological characteristics was statistically analyzed in **Table 1**. The high expression of HMGB3 mRNA was related to tumor size, lymph node metastasis, advanced FIGO stage and depth of cervical invasion (**Table 1**). However, there was no significant correlation between HMGB3 mRNA expression and other clinicopathological features such as age and histology (**Table 1**).

3. HMGB3 promoted CC growth

Loss and gain-of-function experiments were performed to explore the biological function of HMGB3 in CC. We first detected HMGB3 mRNA expression in various CC cell lines by qRT-PCR and the data illustrated that HMGB3 mRNA expression was highly expressed in Hela and CaSki cells lines (both $p<0.001$), while there was relatively less expression in SiHa and C33A cells lines (**Fig. 2A**; $p=0.037$ and $p=0.029$, respectively). Therefore, we used Hela and CaSki cells to knockdown HMGB3, and SiHa and C33A cells to overexpress HMGB3 to perform loss and gain experiments. After transfected with sh-HMGB3, HMGB3 expression was significantly reduced in Hela and CaSki cells compared with sh-NC group (**Fig. 2B**; $p<0.001$). HMGB3 expression increased severely in SiHa and C33A cells when transfected with lentivirus overexpressed of HMGB3 (**Fig. 2C**; $p<0.001$). Then the cell viability was tested with CCK-8 assay and it was found that inhibiting the expression of HMGB3 can effectively reduce cell activity, while overexpression of HMGB3 can increase cell activity in all Hela, CaSki, Siha and C33A cells (**Fig. 2D-G**).

4. Effects of HMGB3 knockdown on cell apoptosis and cell cycle progression in Hela and CaSki cells

Next, we investigated the function of HMGB3 in the cell cycle and apoptosis to study the mechanism underlying the cell viability decrease by HMGB3 knockdown by flow cytometry assay. The results showed that knockdown of HMGB3 blocked HeLa and CaSki cells in the G0/G1 phase (**Fig. 3A and B**; $p=0.027$ and $p=0.014$). At the same time, compared with the sh-NC group, HMGB3 knockdown increased HeLa and CaSki apoptosis (**Fig. 3C**; $p=0.008$ and $p=0.021$). In addition, compared with the sh-NC group, HMGB3 knockdown significantly inhibited the expression of cyclin D3 and cdk2 (**Fig. 3D-F**). These data indicated that HMGB3 promoted the proliferation of CC cells by regulating the cell cycle and apoptosis.

5. Effects of HMGB3 knockdown on HeLa cell growth in vivo

To determine the effect of HMGB3 on the development of CC tumors in vivo, we injected HeLa cells transfected with sh-HMGB31 into nude mice. Our data showed that sh-HMGB3 cell-derived xenograft tumors grew more slowly than sh-NC cell-derived xenograft tumors (**Fig. 4A**). We used qRT-PCR to detect the expression of HMGB3 in tumor tissues. Our data show that the expression of HMGB3 in the sh-HMGB3 group was significantly reduced compared to the sh-NC group (**Fig. 4B**; $p<0.001$). In addition, the average weight of xenograft tumors derived from sh-HMGB3 cells was also significantly less than from sh-NC cells (**Fig. 4C**; $p=0.005$). Moreover, qRT-PCR results showed that the mRNA expression of Ki67 and PCNA, popular proliferation markers, decreased significantly in sh-HMGB3 cell-derived xenograft tumors than those in sh-NC cell-derived xenograft tumors, indicating that sh-HMGB3

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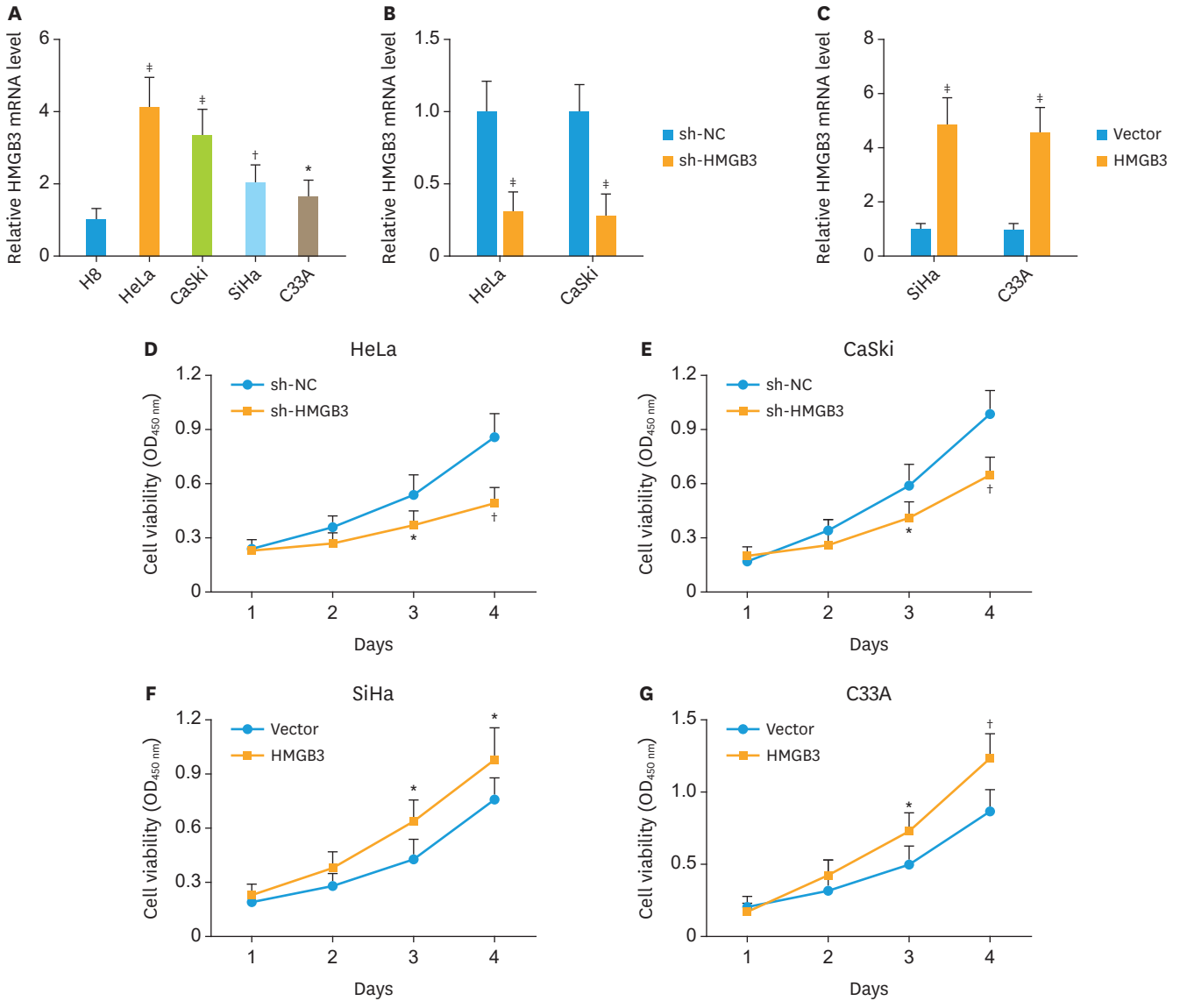

Fig. 2. HMGB3 promotes cervical cancer growth. (A) qRT-PCR was used to analyze the expressions of HMGB3 among different cervical cancer cell lines (H8 was used as negative control). (B) Knockdown efficiency of HMGB3 in Hela and CaSki cells. (C) Overexpression efficiency of HMGB3 in SiHa and C33A cells. (D, E) CCK-8 assay was used to explore the cell viability of Hela and CaSki cells transfected with sh-HMGB3 or sh-NC. (F, G) CCK-8 assay was used to explore the cell viability of SiHa and C33A cells transfected with HMGB3 or vector (n=6–10 for each group). Data are presented as mean±standard deviation. One-way or 2-way analysis of variance analysis followed by post hoc test.
CCK-8, Cell Counting Kit-8; HMGB3, high mobility group box 3; mRNA, messenger RNA; OD, optical density; qRT-PCR, real-time quantitative polymerase chain reaction; sh-HMGB3, shRNAs targeting HMGB3; sh-NC, negative control shRNAs.
*p<0.05, †p<0.01 and ‡p<0.001 compared to their negative control.

could reduce cell proliferation in the CC xenograft (**Fig. 4D and E**; p=0.009 and p=0.022). Therefore, we demonstrated that depletion of HMGB3 inhibited the growth of CC cells in vivo.

6. HMGB3 promoted CC proliferation by regulating Wnt/β-catenin pathway

In order to find the possible mechanism under which cell signaling pathway regulated the progression of CC cells, we used western blot assay to detect the effects of HMGB3 knockdown on the Wnt/β-catenin pathway. Our data showed that HMGB3 inhibition significantly decreased the levels of β-catenin, c-Myc, and MMP-7 in Hela and CaSki cells transfected with sh-HMGB3 (**Fig. 5A-C**). However, overexpression of HMGB3 obviously

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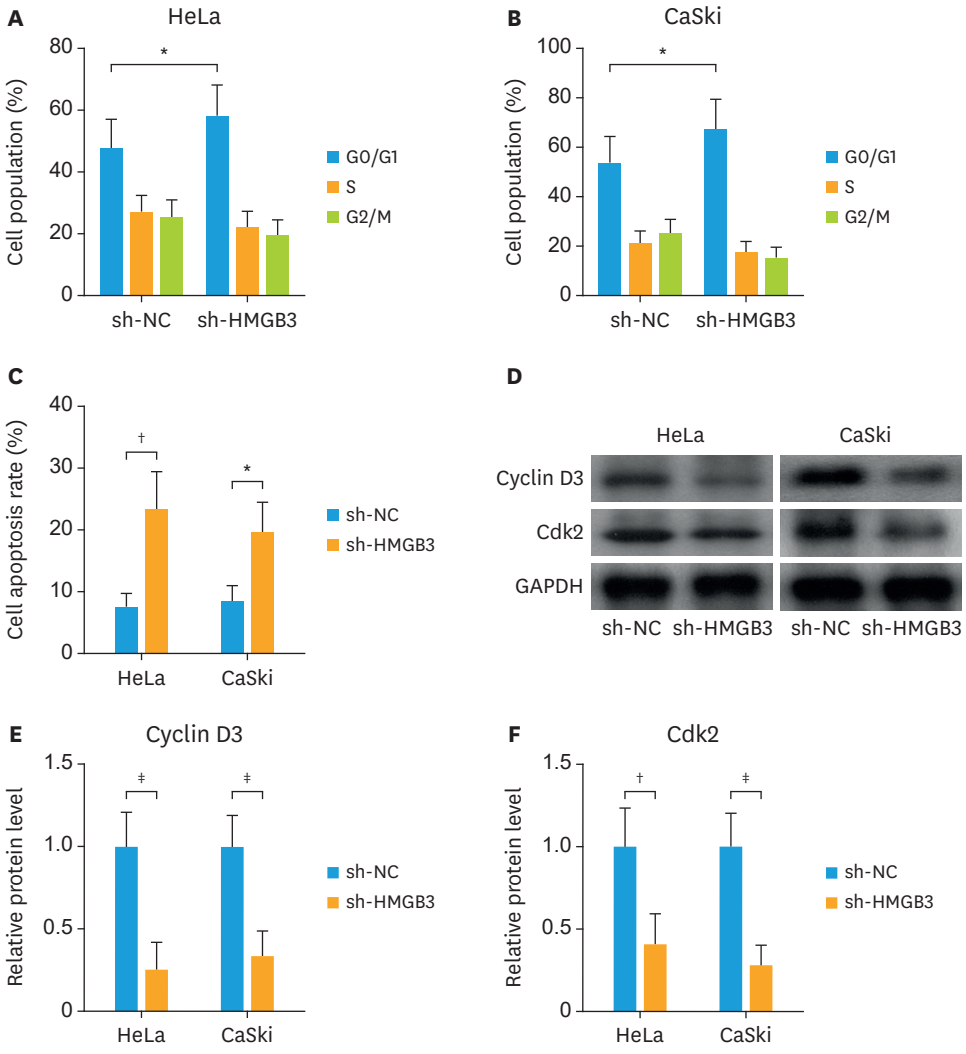

Fig. 3. Effects of HMGB3 knockdown on cell apoptosis and cell cycle progression in Hela and CaSki cells. Flow cytometry was performed to determine the cell cycle (A and B) and apoptosis (C) of HeLa and CaSki cells transfected with sh-HMGB3 or sh-NC. (D) G1 arrest cell cycle markers were analyzed by western blot in HeLa and CaSki cells transfected with sh-HMGB3 or sh-NC and the relative expressions normalized to sh-NC have been quantified (E and F) (n=10 for each group). Data are presented as mean±standard deviation. Two-way analysis of variance analysis followed by post hoc test.
HMGB3, high mobility group box 3; sh-HMGB3, shRNAs targeting HMGB3; sh-NC, negative control shRNAs.
*$p<0.05$, †$p<0.01$ and ‡$p<0.001$ between the indicated groups.

increased the protein levels of c-Myc and MMP-7 in SiHa and C33A cells while transfection with sh-β-catenin reversed the increase (**Fig. 5D-F**). These data suggested that Wnt/β-catenin pathway might participate in the HMGB3-induced proliferation and metastasis of CC cells.

DISCUSSION

CC is the most common gynecological malignancy in the world. The incidence of carcinoma in situ is high in women with 30 to 35 years of age, and that of invasive cancer in women with 45 to 55 years. In recent years, CC tends to be developed in younger age and almost 90% of deaths from CC occur in developing countries. However, the mechanism of this disease is

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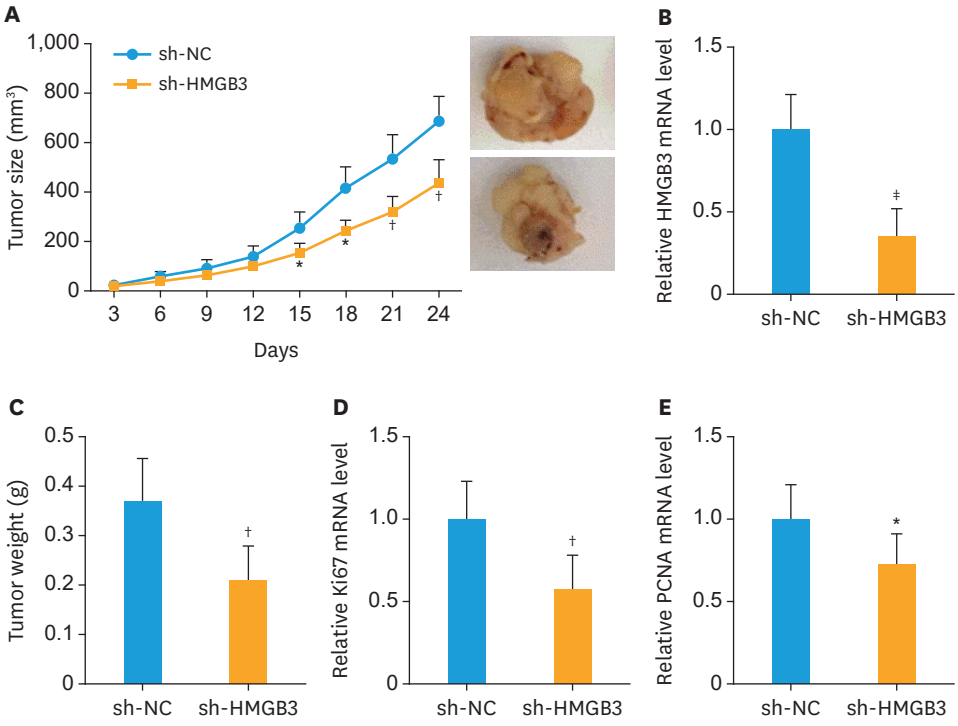

Fig. 4. Effects of HMGB3 knockdown on HeLa cell growth in vivo. (A) Representative images of the xenograft tumors from HeLa cells transfected with sh-HMGB3 or sh-NC after 24 days and tumor growth curves determined every 3 days after injection. (B) The relative expression levels of HMGB3 in tumor tissues obtained from mice after 24 days of injection were detected by qRT-PCR. (C) The tumor weight was measured after 24 days of injection. (D, E) qRT-PCR was used to determine the expressions of Ki67 and PCNA in tumor obtained from mice after 24 days of injection (n=6–10 for each group). Data are presented as mean±standard deviation. t-tests followed by Mann-Whitney test. HMGB3, high mobility group box 3; qRT-PCR, real-time quantitative polymerase chain reaction; sh-HMGB3, shRNAs targeting HMGB3; sh-NC, negative control shRNAs.
*$p<0.05$, †$p<0.01$ and ‡$p<0.001$ compared to their negative control.

still unclear. HMGB3 plays an important role in development of many cancers, and it has been reported to participate in the proliferation and migration of tumor cells [12-15]. In order to systematically study the role of HMGB3 in the growth of CC, we first analyzed the expression of HMGB3 in tumor samples from patients, and then modulated HMGB3 in CC cell lines to study its effect on CC proliferation and migration.

First, we detected HMGB3 expression in CC tissues from clinical patients. The results confirmed that HMGB3 expression both in mRNA and protein levels in 58 CC tissues was higher compared to paired adjacent non-tumor tissues. Furthermore, we found that HMGB3 mRNA expression was significantly higher in patients with bigger tumor size (≥4 cm) and advanced FIGO stage, indicating that HMGB3 was involved in CC carcinogenesis. But the results showed that there were no significant correlations between HMGB3 mRNA expression and other clinical pathologic features like age and histology. These results were consistent with other studies that HMGB3 expression was higher in cancer tissues compared to normal tissues nearby [13-16].

Next, we used loss-of-function and gain-of-function experiments to explore the biological function of HMGB3 in CC. The lentivirus overexpressing HMGB3 were transfected into SiHa and C33A cell lines, which have a low expression of HMGB3 by themselves. Sh-HMGB3 plasmids were transfected into Hela and CaSki cell lines which have high expression of HMGB3 by

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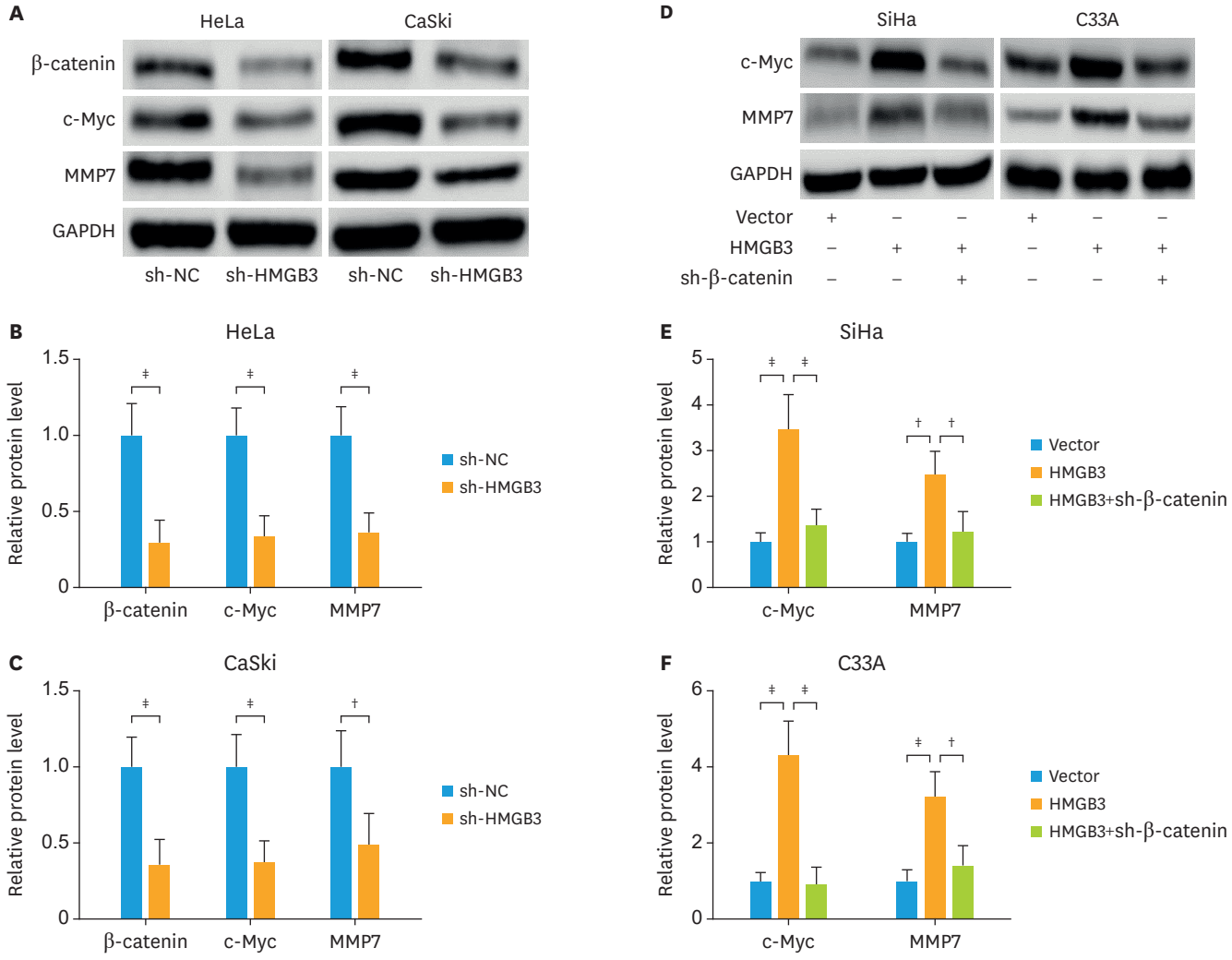

Fig. 5. HMGB3 promotes cervical cancer proliferation by regulating Wnt/β-catenin pathway. (A) Western blot was performed to detect β-catenin, c-Myc, and MMP-7 expression after HMGB3 knockdown in HeLa and CaSki cells and relative expressions were normalized to sh-NC (B and C). (D) The expression of c-Myc, MMP-7 in SiHa and C33A cells were detected by western blot after increasing the expression of HMGB3 or increasing the expression of HMGB3 but inhibited β-catenin expression. GAPDH was used as loading control and relative expressions were normalized to vector control (E and F) (n=10 for each group). Data are presented as mean±standard deviation. Two-way analysis of variance analysis followed by post hoc test.
GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HMGB3, high mobility group box 3; MMP-7, matrix metalloproteinase-7; sh-β-catenin, shRNAs targeting β-catenin; sh-HMGB3, shRNAs targeting HMGB3; sh-NC, negative control shRNAs.
*p<0.05, †p<0.01 and ‡p<0.001 between the indicated groups.

themselves. We found that HMGB3 knockdown can effectively reduce cell activity, while HMGB3 overexpression can increase cell activity, indicating that HMGB3 promoted CC growth. HMGB3 knockdown could arrest HeLa and CaSki cells at G0/G1 phase and increase cell apoptotic events by inhibiting the expression of cell cycle proteins like cyclin D3 and cdk2, meaning that HMGB3 could promote the proliferation phenotype of CC cells. Another study showed that HMGB3 overexpression was related to poor prognosis of resected gastric adenocarcinoma and HMGB3 RNA knockdown inhibited the growth of gastric cancer cells by regulating cell cycle distribution [16]. In bladder cancer, upregulation of HMGB3 is also associated with poor prognosis. It is also found that HMGB3 enhances cell proliferation and migration in bladder cancer cell lines and proliferation and invasion in breast cancer cell lines [21,22].

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Furthermore, we built a sh-HMGB3 cell-derived xenograft nude mice model by injecting HeLa cells transfected with sh-HMGB3 to detect the effect of HMGB3 on CC tumorigenesis in vivo. The mice with sh-HMGB3 cell-derived xenograft tumors grew slowly and had smaller tumor, and HMGB3 expression in tumor tissues was lower than those mice with sh-NC cell-derived xenograft tumors. Ki67 is a popular proliferation marker and is routinely used in pathology examinations due to its diagnostic and prognostic power in cancer. PCNA is another common marker, yet multiple studies have shown that Ki67 is more sensitive and specific when evaluating cell proliferation in tumors from various origins. We found that both Ki67 and PCNA mRNA expressions decreased significantly in sh-HMGB3 cell-derived xenograft tumors than those in sh-NC cell-derived xenograft tumors, verifying that HMGB3 depletion also suppressed CC cell growth in vivo.

The regulation of Wnt/β-catenin activity is closely related to the development of a variety of cancers [23,24]. Wnt/β-catenin signaling activates β-catenin to initiate transcription of its downstream target genes and has been reported to be involved in the occurrence and development of CC [25-27]. In the canonical pathway, Wnt activation disrupts the destruction complex, allowing β-catenin to accumulate and subsequently translocate to the nucleus, where it interacts with transcription factors to increase expression of oncogenes, such as c-Myc and CyclinD1 [28,29]. Our results showed that HMGB3 inhibition significantly decreased the levels of β-catenin, c-Myc and MMP-7 in Hela and CaSki cells transfected with sh-HMGB3, while overexpression of HMGB3 obviously increased the protein levels of c-Myc and MMP-7 in SiHa and C33A cells when transfection with sh-β-catenin, indicating that HMGB3 may promote CC proliferation by regulating WNT/β-catenin pathway. Recent study has shown that HMGB3 plays a carcinogenic role in colorectal cancer and also verified that HMGB3 promotes growth and migration in colorectal cancer by regulating Wnt/beta-catenin pathway [28]. Another study demonstrated that HMGB3 was a direct target of miR-758, and HMGB3 overexpression rescued the viability, proliferation, invasion and migration of HeLa cells reduced by an miR-758 mimic. Further experiments showed that HMGB3 overexpression enhanced the expression of β-catenin and its target genes MMP-7 and c-Myc. These results illustrated that microRNA-758 inhibited CC cell proliferation and metastasis by targeting HMGB3 through the Wnt/β-catenin signaling pathway [30]. All these studies were consistent with our results, and our study gave a further understanding of the role of HMGB3 in CC.

In summary, the present study demonstrated that HMGB3 was overexpressed in CC tissues from clinical patients. Knockdown of HMGB3 can effectively reduce cell activity, while overexpression of HMGB3 can increase cell activity. HMGB3 knockdown increased cell apoptotic events by arresting HeLa and CaSki cells at G0/G1 phase, indicating that HMGB3 could promote the proliferation of CC cells. As a conclusion, these data showed that HMGB3 functioned as a tumor promotor, it promoted CC growth by regulating Wnt/β-catenin pathway. This study provided new insight into the mechanisms underlying CC development and suggested that targeting HMGB3 may represent a promising therapeutic strategy for CC treatment.

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