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Original contribution

HMGB2 promotes the malignancy of human gastric cancer and indicates poor survival outcome☆,☆☆

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Summary HMGB2 is an important protein in carcinogenesis. However, little is known about the specific role of HMGB2 in gastric cancer. In the present study, HMGB2 expression was evaluated in 198 primary gastric cancer tissues and their adjacent nontumor controls. The correlation between HMGB2 expression and clinico-pathological features and survival was assessed. The effect of HMGB2 on cell proliferation, invasion, and glycolysis was examined in vitro. The expression of HMGB2 was significantly increased in human gastric cancer when compared with nontumor tissues ($P < .001$). High HMGB2 expression correlated with large tumor size ($P = .001$), advanced T stage ($P = .007$), and presence of lymph node metastasis ($P = .004$). Moreover, high HMGB2 expression was validated as an independent prognostic factor in both univariate and multivariate analyses ($P < .05$). Experimentally, silencing HMGB2 expression by stable transfected shRNA significantly decreased the proliferation, invasion, and glycolysis of gastric cancer cells. In conclusion, HMGB2 is a novel prognostic biomarker for survival in gastric cancer, and knockdown HMGB2 expression in gastric cancer cells attenuated proliferation and invasion, and impaired glycolysis in gastric cancer cells. Hence, HMGB2 may serve as a new biomarker and a potential therapeutic target in gastric cancer.

1. Introduction

Gastric cancers is one of the deadliest malignancies, ranking as the fourth most common cancer in the world [1] and the second most common cancer in China [2]. Despite recent improvements in multidisciplinary strategies, the prognosis of patients with gastric cancer remains dismay. About 20% to 50% patients will suffer local recurrences or distant metastases after gastrectomy [3-5]. Recurrences and metastases are main reasons for cancer-related death in patients after gastrectomy [6]. In fact, the development of metastases and recurrences involves the accumulation of multiple genetic and epigenetic changes in critical genes that control cell proliferation and migration [7]. Understanding the mechanisms that underline progression and metastasis may help in predicting prognosis and designing treatment strategies.

High-mobility group box (HMGB) proteins are ubiquitous and the second most abundant proteins, and exert global genomic functions in establishing active or inactive chromatin domains [8,9]. HMGB1 and HMGB2 are highly conserved (with >80% amino acid identity) and have indistinguishable biological properties such as binding to DNA without sequence specificity [9-12]. HMGB1 has been reported to be an oncogene in gastric cancer. High HMGB1 is associated with poorer prognosis in patients with gastric cancer [13,14]. Knockdown of HMGB1 suppresses growth and invasion of gastric cancer

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cells through the NF-κB pathway in vitro and in vivo [15]. Ectopic HMGB1 expression contributes to tumor angiogenesis through interleukin 8 in vitro [16]. HMGB2 has been validated as a downstream target of miR-23b-3p and lncRNA MALAT1 [17,18]. However, the clinical significance HMGB2 in gastric cancer has not been fully studied. Hence, we performed the present study to investigate the significance of HMGB2 expression in gastric cancer by immunohistochemistry (IHC) study. Furthermore, we analyzed the effects of HMGB2 on cell growth, invasion, and Warburg effect, which was thought as fundamental for tumor growth and progress, in vitro. Our studies indicated that high HMGB2 predicted poor prognosis by promoting cell proliferation, invasion, and glycolysis in gastric cancer.

2. Materials and methods

2.1. Patients and tissue samples

The study was approved by the institutional review boards of The First People's Hospital of Shangqiu. Informed consent was obtained from all participants, and all patient-derived specimens were collected and archived under protocols approved. A total of 198 gastric cancer and their adjacent nontumor controls samples were collected. The nontumor control samples were defined as at least 15 cm from the tumor edges.

The inclusion criteria were as follows: (i) having a distinctive pathologic diagnosis of gastric cancer; (ii) surgical resection, defined as complete resection of primary tumor and regional lymph node, with the cut margin being free of cancer by pathological examination; and (iii) having intact follow-up. The exclusion criteria included (i) having distant metastases and (ii) having anticancer treatment before surgical resection.

To further confirm the prognostic value of HMGB2 in gastric cancer, an external validation cohort provided by Professor Jiang at Yancheng No. 1 Peoples' Hospital was used. The detailed information of the cohort was included in a previous study [19].

2.2. Immunohistochemistry

Tissue samples were fixed in 4% formalin and embedded in paraffin. IHC staining was performed as described previously

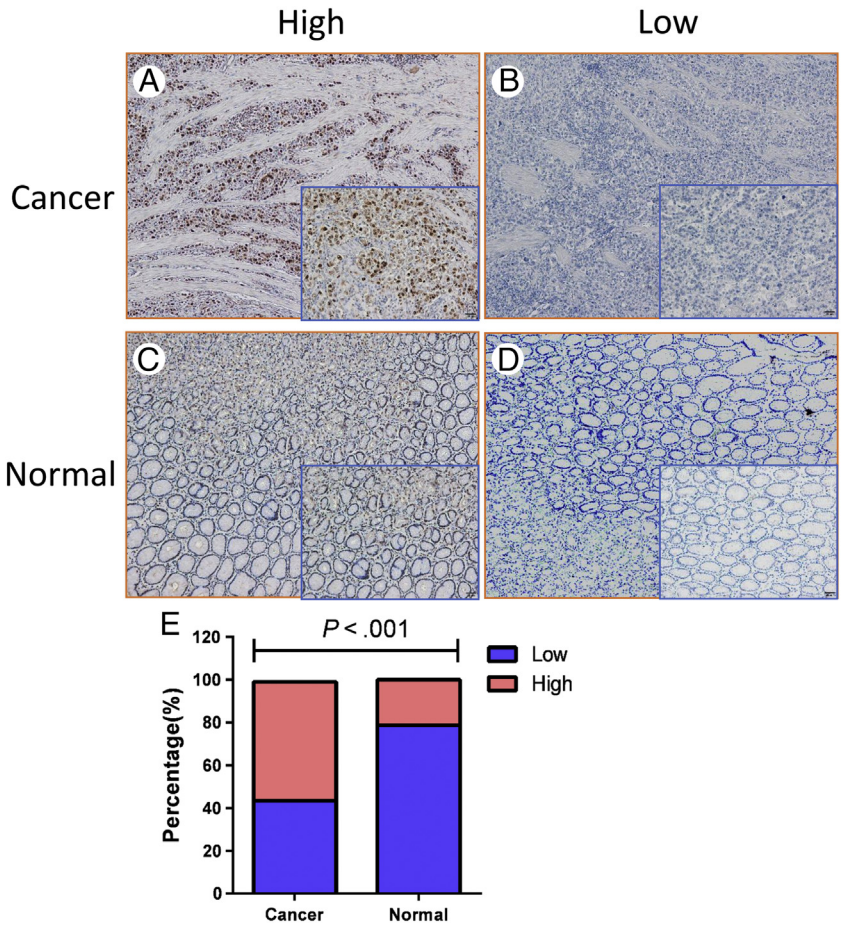

Fig. 1 HMGB2 expression in gastric cancer. HMGB2 expression was detected by IHC staining in gastric cancer and paired nontumor tissues. HMGB2 was mainly localized within the nucleus of cancer cells. Examples of high (A) or low (B) HMGB2 expression in gastric cancer, and high (C) or low (D) HMGB2 expression in nontumor gastric tissues. As visualized in a ×100 and ×400 magnifications (insets). E, The percentage of high HMGB2 expression was significantly higher in gastric cancer than in their nontumor controls ($P < .001$).

[20]. HMGB2 antihuman rabbit antibody was used at a dilution of 1:100 (14597-Q3 1-APl; Proteintech, Wuhan, China); phosphate-buffered saline was used as a negative control. The results were assessed by 2 independent single-blinded pathologists. A semiquantitative scoring system [21] was used to evaluate both staining intensity (0, no staining; 1+, weak staining; 2+, moderate staining; 3+, strong staining) and the percentage of stained cells (0, <5%; 1, 5%-25%; 2, 26%-50%; 3, 51%-75%; and 4, >75%). The scores for staining intensity and percentage of positive cells were then multiplied to generate the immunoreactivity score for each case [22]. All cases were sorted into 2 groups according to the immunoreactivity score. High expression of HMGB2 was defined as detectable immunoreactions in the nucleus with an immunoreactivity score of 4 or higher.

2.3. Cell culture

The human gastric cancer cell lines AGS and MGC803 were used for functional study. The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum in a humidified 37°C incubator supplemented with 5% CO_2.

2.4. Plasmids and the establishment of stable transfection cell lines

shRNA targeting the *HMGB2* gene (GCTCAATACTAGCTTCAGTAT) was inserted into pLKO.1 plasmid. Scramble sequence (5′-TTCTCCGAACGTGTCACGT-3′) was used as a negative control. AGS and MGC803 were transfected with the pLKO.1-shHMGB2 expression vector and pLKO.1-scramble.Transfected cells were selected using puromycin after the cells were transfected with knockdown vector or control plasmids.

2.5. Quantitative real-time polymerase chain reaction

Total RNA was extracted from tissues and cells using a TRIzol reagent (Invitrogen, Carlsbad, USA), and then microRNAs were reverse transcribed to cDNA using a reverse transcription kit (RR036A; Takara, Tokyo, Japan). Quantitative real-time polymerase chain reaction (RT-PCR) was performed using a SYBR-Green PCR kit on an ABI 7900 Fast Real-Time PCR system. The expression of the target gene messenger RNA was normalized to β-actin. All experiments were carried out in triplicate. The relative quantity (RQ) value was used to calculate the relative expression of the genes.

2.6. Western blotting

Equal amounts of cell lysates were subjected to 10% SDS-PAGE, and proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Ireland). The membranes were probed overnight with specific primary antibodies HMGB2 (14597-1-APl; Proteintech) and β-actin (Abcam, Cambridge, UK; ab133626, 1:5000) overnight at 4°C, which were detected with corresponding secondary antibodies (Cell Signaling Technology, Danvers, USA). The immunoreactive bands were visualized using enhanced chemiluminescence (Thermo Scientific, Carlsbad, USA).

2.7. Cell proliferation assay

Cells were seeded and cultured in 96-well plates. A CCK-8 assay (Dojindo, Japan) was performed, and the optical density at 450 nM was measured in an automatic microplate reader (Bio Tek, Winooski, USA). Each experiment was performed in triplicate and repeated at least twice. The data are presented as the average ± SD.

Table 1 Association between HMGB2 expression and clinicopathological factors in gastric cancers

Characteristics	Total	HMGB2 expression		*P*
		Low expression	High expression	
Sex				.504
Male	102	43 (48.9)	59 (53.6)	
Female	96	45 (51.1)	51 (46.6)	
Age (y)				.702
<60	105	48 (54.5)	57 (51.8)	
≥60	93	40 (45.5)	53 (48.2)	
Histologic grade				.442
G1/G2	87	36 (40.9)	51 (46.4)	
G3	111	52 (59.1)	59 (53.6)	
Tumor diameter (cm)				<.001 *
<5	96	65 (73.9)	31 (28.2)	
≥5	102	23 (26.1)	79 (71.8)	
Subtype				.662
Intestinal	142	65 (45.8)	77 (54.2)	
Diffuse	19	9 (47.4)	10 (52.6)	
Mixed	37	14 (37.8)	23 (62.2)	
T stage				.007 *
T1	3	3 (3.4)	0 (0)	
T2	34	22 (25.0)	12 (10.9)	
T3	97	41 (46.6)	56 (50.9)	
T4	64	22 (25.0)	42 (38.2)	
N stage				.004 *
N0	61	30 (34.1)	31 (28.2)	
N1	51	31 (35.2)	20 (18.2)	
N2	56	20 (22.7)	36 (32.7)	
N3	30	7 (8.0)	23 (20.9)	
Lymphovascular invasion				.495
Negative	153	70 (79.5)	83 (75.5)	
Positive	45	18 (16.2)	27 (26.5)	
Perineural invasion				.182
Negative	146	69 (78.4)	77 (70.0)	
Positive	52	19 (21.6)	33 (30.0)	

* $P < .05$.

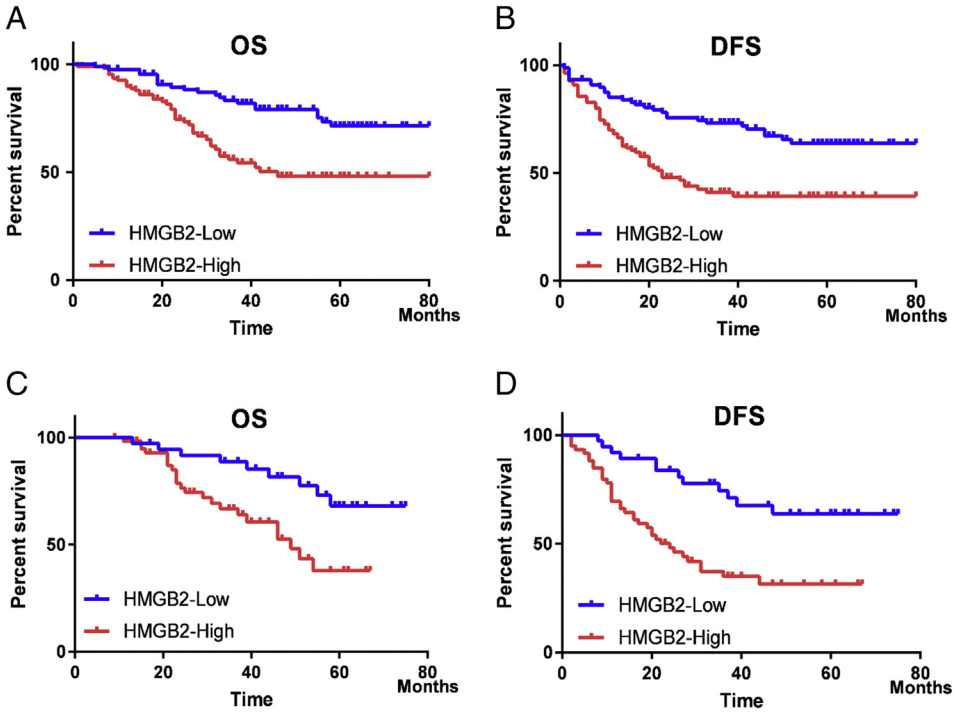

Fig. 2 High HMGB2 expression is correlated with poor survival for gastric patients. Patients were then divided into HMGB2 high and low expression groups according to immunoreactivity score. Kaplan-Meier analyses were performed to evaluate the correlation of HMGB2 expression and patients' survival. In the discovery cohort, high HMGB2 correlated with poor OS ($P < .001$; A) and DFS ($P < .001$; B). In the validation cohort, patients with high HMGB2 expression also had significantly shorter OS (37.9% versus 68.1%, $P = .006$; C) and DFS (31.3% versus 63.6%, $P = .001$; D) when compared with those with low HMGB2 expression.

2.8. Cell invasion assay

Cells in serum-free medium (200 μL containing 5×10^4 cells) were added to upper Transwell chambers (Corning, New York, USA) with an 8-mm pore size. The bottom chamber contained medium with 10% fetal bovine serum as a chemoattractant. After a 24-hour incubation at 37°C, the cells on the upper surface of the membrane were removed with a cotton swab. The cells on the lower surface were fixed in ethanol and stained with 0.05% crystal violet. Cell motility was quantified by counting the number of cells that had migrated to the lower surface of the membrane. For each membrane, 5 random fields were counted using a light microscope at ×100 magnification, and the mean value for each membrane was calculated.

2.9. Glycolysis analysis

Glucose Uptake Colorimetric Assay Kit (Biovision, Milpitas, CA) and Lactate Colorimetric Assay Kit (Biovision) were purchased to examine the glycolysis process in gastric cancer

Table 2 Univariate and multivariate Cox proportional hazards analyses of the expression of HMGB2 gene and OS in patients with gastric cancer

Factor	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Sex	0.934 (0.570-1.531)	.785		
Age	1.000 (0.979-1.021)	.994		
Diameter	1.217 (0.741-1.997)	.438		
Subtype	1.201 (0.897-1.607)	.220		
T stage	1.905 (1.319-2.750)	.001 *	1.576 (1.006-2.470)	.047 *
N stage	1.613 (1.265-2.055)	<.000 *	1.332 (1.016-1.747)	.038 *
Grade	1.363 (0.819-2.269)	.233		
Lymphovascular invasion	2.420 (1.438-4.073)	.001 *	1.826 (1.080-3.088)	.025 *
Perineural invasion	1.854 (1.104-3.113)	.020 *	2.124 (1.229-3.673)	.007 *
Tumor location	0.927 (0.724-1.186)	.546		
HMGB2	2.621 (1.539-4.464)	<.001 *	1.972 (1.138-3.416)	.015 *

* $P < .05$.

cells according to the manufacturer's protocol. Real-time PCR was performed to test expression of glycolytic enzymes. All reactions were run in triplicate.

2.10. Statistical analysis

Statistical analyses were performed using SPSS 21.0 statistical package (SPSS, Chicago, IL). Differences between groups and correlations between HMGB2 expression and the clinicopathological features of patients with gastric cancer were evaluated using χ^2 tests or Fisher exacts test. The 5-year overall survival (OS) and disease-free survival (DFS) were calculated using the Kaplan-Meier method to estimate the survival and hazard functions of censored data. The difference in survival between the groups at each observed event time was compared by the log-rank test. The in vitro study was analyzed by 1-way analysis of variance. A P value less than .05 was considered statistically significant.

3. Results

3.1. HMGB2 is highly expressed in gastric cancer samples

In an attempt to investigate whether HMGB2 was involved in gastric cancer, IHC study of HMGB2 protein expression in 198 paired gastric cancer samples and adjacent nontumor tissue was analyzed. HMGB2 was mainly stained in nucleus of gastric cancer cell and gastric epithelium cells. Representative images of HMGB2 in gastric cancer and nontumor tissue are presented in Fig. 1A. It was observed that HMGB2 was highly expressed in 55.6% (110/198) of gastric cancer samples, which were markedly higher than that in their nontumor tissue controls (Fig. 1B).

3.2. Correlation between HMGB2 expression and clinicopathological parameters

The relationship between HMGB2 protein expression levels and patients' clinicopathological parameters is demonstrated in Table 1. High HMGB2 expression was significantly correlated with large tumor size ($P < .001$), advanced T stage ($P = .007$), and presence of lymph node metastasis ($P = .004$). However, no significant association was identified between HMGB2 expression and the other clinicopathological characteristics, including sex ($P = .504$), age ($P = .704$), tumor grade ($P = .442$), lymphovascular invasion ($P = .495$), and perineural invasion ($P = .182$).

3.3. High HMGB2 expression predicts poor prognosis

Of the 198 cases, tumor recurred in 90 patients (45.5%), and 63 (31.8%) patients died of the disease during the follow-up. Patients with high expression of HMGB2 had a significantly shorter DFS and OS when compared with those with low expression (both log-rank test, $P < .001$; Fig. 2A and B). In univariate analysis, T stage (hazard ratio, 1.905; 95% confidence interval [CI], 1.319-2.750; $P = .001$), N stage (HR, 1.613; 95% CI, 1.265-2.055; $P < .001$), lymphovascular invasion (HR, 2.420; 95% CI, 1.438-4.073; $P = .001$), perineural invasion (HR, 1.854; 95% CI, 1.104-3.113; $P = .020$), and HMGB2 expression (HR, 2.621; 95% CI, 1.539-4.464; $P < .001$) were associated with OS, whereas T stage (HR, 1.984; 95% CI, 1.454-2.708; $P < .001$), N stage (HR, 1.489; 95% CI, 1.225-1.810; $P < .001$), lymphovascular invasion (HR, 1.556; 95% CI, 1.010-2.397; $P = .045$), perineural invasion (HR, 2.502; 95% CI, 1.616-3.8749; $P < .001$), and HMGB2 expression (HR, 2.390; 95% CI, 1.526-3.742; $P < .001$) were associated with DFS. In multivariate analysis, incorporated factors that were significant in univariate analysis further confirmed that HMGB2 was an independent

Table 3 Univariate and multivariate Cox proportional hazards analyses of the gene expression of HMGB2 and DFS in patients with gastric cancer

Factor	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P	HR (95% CI)	P
Sex	1.035 (0.684-1.565)	.871		
Age	0.899 (0.594-1.361)	.615		
Diameter	1.352 (0.890-2.052)	.157		
Subtype	1.165 (0.910-1.492)	.225		
T category	1.984 (1.454-2.708)	<.001 *	1.566 (1.091-2.248)	.015 *
N stage	1.489 (1.225-1.810)	<.001 *	1.159 (0.930-1.445)	.188
Grade	1.556 (1.010-2.397)	.045 *	1.339 (0.860-2.083)	.196
Lymphovascular invasion	2.502 (1.616-3.874)	<.001 *	2.015 (1.290-3.150)	.002 *
Perineural invasion	1.462 (0.931-2.296)	.099		
Tumor location	0.920 (0.747-1.134)	.434		
HMGB2	2.390 (1.526-3.742)	<.001 *	1.970 (1.239-3.132)	.004 *

* $P < .05$.

prognostic factor for both OS (HR, 1.972; 95% CI, 1.138-3.416; $P = .015$) and DFS (HR, 1.970; 95% CI, 1.239-3.132; $P = .004$; Tables 2 and 3).

To further confirm the prognostic value of HMGB2 in gastric cancer, an external validation including 96 patients diagnosed as having gastric cancer were used. All patients underwent radical surgery. Survival analysis indicated that patients with high HMGB2 expression had significantly shorter DFS (31.3% versus 63.6%, $P = .001$) and OS (37.9% versus 68.1%, $P = .006$) when compared with those with low HMGB2 expression (Fig. 2C and D).

3.4. Silencing HMGB2 expression decreases proliferation and invasion of gastric cancer cells

As HMGB2 expression correlated with tumor size and advanced tumor stage, it was hypothesized that knockdown HMGB2 expression could impair proliferation and invasion of gastric cancer cells. The stable HMGB2 knockdown in AGS and MGC803 cells was established. RT-PCR and Western blot analysis were performed to verify that HMGB2 was successfully silenced at both transcriptional and protein levels in AGS and MGC803 cells (Fig. 3A and B). CCK-8 analysis

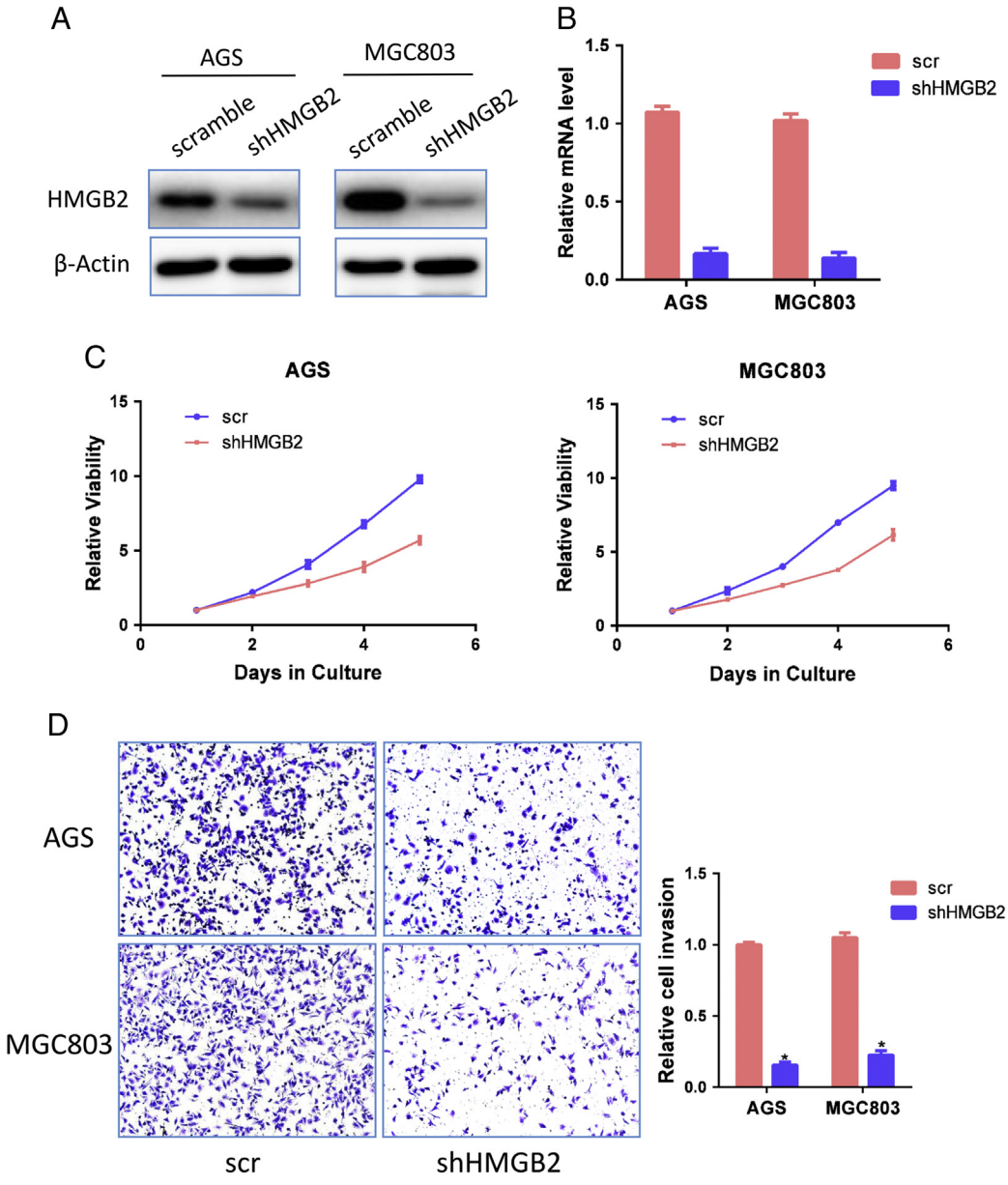

Fig. 3 Silencing HMGB2 expression decreased proliferation and invasion of gastric cancer cells. The stable HMGB2 knockdown in AGS and MGC803 cells was established. Western blot (A) and RT-PCR analysis (B) were performed to verify that HMGB2 was successfully silenced at both transcriptional and protein levels in AGS and MGC803 cells. C, CCK-8 analysis indicated that silencing HMGB2 expression significantly decreased cell proliferation in AGS and MGC803 cells. D, Transwell study demonstrated that knockdown HMGB2 expression significantly impaired cell invasion abilities. *$P < .05$.

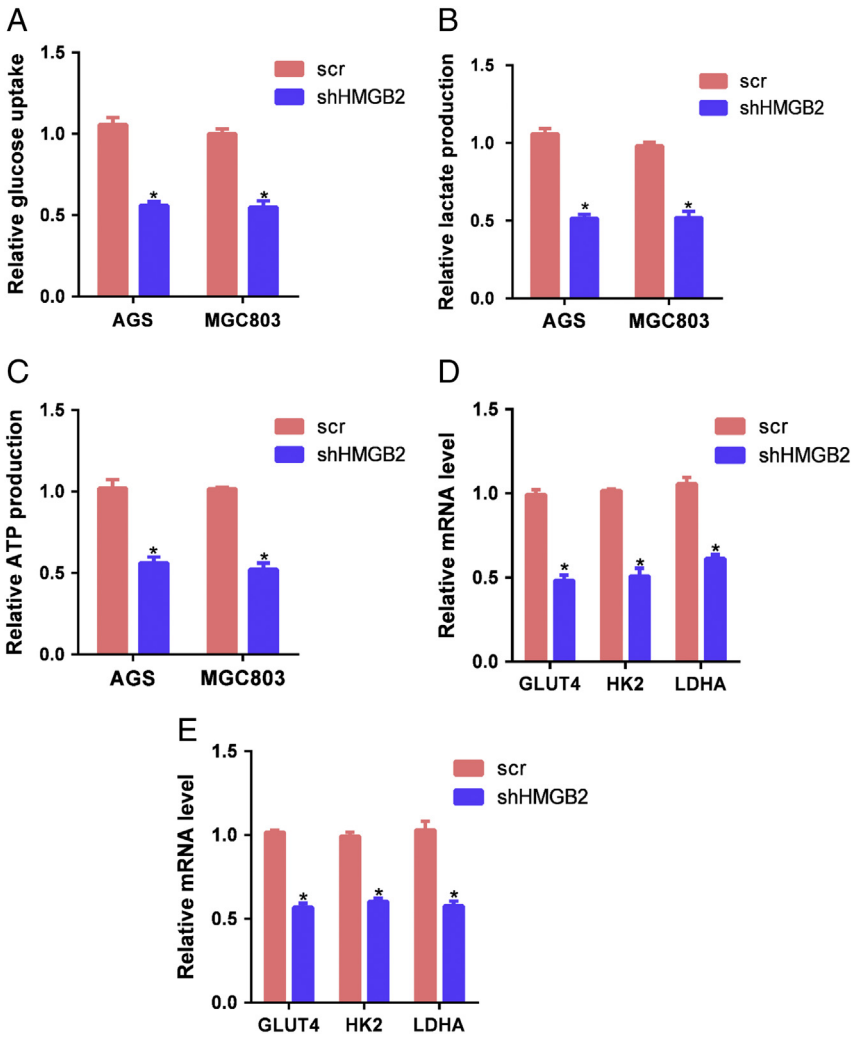

Fig. 4 Silencing HMGB2 expression impaired glycolysis of gastric cancer cells. Silencing HMGB2 expression strongly decreased glucose consumption (A), lactate production (B), and ATP production (C) in AGS and MGC803 cells. D and E, RT-PCR analysis demonstrated that knockdown HMGB2 expression significantly decreased GLUT4, HK2, and LDHA expression in AGS (D) and MGC803 cells (E). *$P < .05$.

indicated that silencing HMGB2 expression significantly decreased cell proliferation in AGS and MGC803 cells (Fig. 3C). Transwell study demonstrated that knockdown HMGB2 expression significantly impaired cell invasion abilities (Fig. 3D).

3.5. Silencing HMGB2 expression impairs glycolysis of gastric cancer cells

Glucose metabolism reprogram to glycolysis is fundamental for cancer cell growth and progression. We then examined glucose consumption, lactate production, and ATP production in HMGB2 knockdown cells. As anticipated, silencing HMGB2 expression strongly decreased glucose consumption, lactate production, and ATP production in AGS and MGC803 cells (Fig. 4A-C). Glycolysis is a multistep enzymatic reaction involved with a series of rate-limiting enzymes. As demonstrated in Fig. 4D and E, knockdown HMGB2 expression up-regulated or down-regulated several rate-limiting enzymes in glycolysis process, with most significant being GLUT4, HK2, and LDHA.

4. Discussion

Studies on prognosis of patients with gastric cancer and on prognostic factors to predict the risk of recurrence and metastasis in gastric cancer patients are intriguing and could affect clinical practice. Although the TNM stage has been regarded as the most powerful predictor for survival, the survival of patients is quite different, and even patients diagnosed at the same stage are spited into death or long time survival subgroup. Some established biomarkers such as c-MET [23], HER-2 [24], and AFP [25] have already been proven to play significant roles in prognosis and selection of patients for personalized therapy. As such, it is urgent to improve the

management of patients and understand the biology of gastric cancer. In the present study, we mainly focused on HMGB2 and found that HMGB2 expression levels were significantly higher in cancer tissues when compared with their nontumor controls. Moreover, high HMGB2 expression correlated with inferior clinicopathological parameters, such as large tumor size, advanced T stage, and presence of lymph node metastases. Importantly, HMGB2 was validated as an independent prognostic factor for gastric cancer in both univariate and multivariate survival analyses. These data strongly suggested that HMGB2 may act as an oncogene in gastric cancer tumorigenesis and progression.

To better unveil the role of HMGB2 in gastric cancer, functional studies were used for further analysis. As anticipated, knockdown HMGB2 expression significantly decreased proliferation and invasiveness of human gastric cancer cells. Kwon et al [10] investigated the expression of HMGB2 in patients with hepatocellular carcinoma and found that HMGB2 overexpression was significantly correlated with shorter OS, and knockdown HMGB2 expression decreased cell proliferation. Wu et al [26] demonstrated that HMGB2 protein expression was significantly higher in glioblastoma multiforme than in controlled brain tissues. High HMGB2 expression was the only independent prognostic factor for OS in a multivariate analysis. Silencing HMGB2 expression attenuated cell viability and invasion in vitro and significantly decreased tumor volume in vivo. Thus, it is not surprising that HMGB2 is negatively associated with survival and promotes proliferation and invasion in gastric cancer.

Unlike differentiated normal cells, tumor cells have an unrestricted capacity to divide and proliferate. Relentless biosynthesis of macromolecules that are needed for the growth of newly divided cells requires the uptake of glucose and other carbon sources in excess of energetic needs [27-29]. Most tumor cell types display modified rates and pathways of energetic and anabolic metabolism in comparison to their tissue of origin [30]. The aerobic glycolysis, which is first named by Warburg, is a shift from oxidative phosphorylation to glycolysis, a feature of which is increasing lactate production even at normal oxygen concentrations, and is regarded as the root of cancer development and progression [29,31,32]. Because HMGB2 correlated with large tumor size and advanced TN stage, and promoted proliferation and invasion in gastric cancer, we questioned whether the effect was the results of glucose metabolism transformation. As expected, knockdown HMGB2 expression significantly decreased glucose uptake, lactate secretion, and ATP production in gastric cancer cells. Silencing HMGB2 inhibited HIF1α-mediated glycolysis process in pancreatic cancer [8]. HMGB2 promoted metabolic reprogramming process in breast cancer cells by targeting LDHB and FBP1 [9]. Thus, it may universal phenomenon that HMGB2 promotes glycolysis in cancer.

There were some limitations in the present study. First, no in vivo experiments using animals were performed to observe the effects of HMGB2 on progression and metastases. Second, the formation and progression of gastric cancer is affected by many tumor suppressors and oncogenes, so biomarkers from a single gene may not sufficient [33]. Third, although we found that knockdown HMGB2 expression could significantly decrease GLUT4, HK2, and LDHA expression, further study is needed to explore the detailed mechanism.

In conclusion, we found that HMGB2 was a novel prognostic biomarker for survival in gastric cancer, and silencing HMGB2 expression significantly attenuated proliferation and invasion and decreased glycolysis rate of gastric cancer cells. Hence, HMGB2 may serve as a new biomarker and a potential therapeutic target in gastric cancer treatment.

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