



HMGB2 upregulation promotes the progression of hepatocellular carcinoma cells through the activation of ZEB1/vimentin axis

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Background: High mobility group box 2 (HMGB2) is abnormally expressed in human cancers and participated in multiple biological behaviors, such as proliferation, invasion and prognosis. However, its role in hepatocellular carcinoma (HCC) is largely unknown.

Methods: In clinical sample analysis, 62 HCC patients were enrolled in this study. The expression of HMGB2 was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemical method, clinical prognosis data were analyzed by Kaplan-Meier analysis. In cellular and molecular biology experiments, HMGB2 expression was analyzed in HCC cells. HMGB2 knockdown model was constructed by small interfering RNA (siRNA). Cell counting kit-8 (CCK-8) and cell migration & invasion assay were used to evaluate cell proliferative potential and motility. Recombinant human vimentin protein was used to partially restore the expression and function of vimentin. Western blot and immunochemical staining were performed to detect HMGB2 protein, zinc finger E-box binding homeobox 1 (ZEB1) and vimentin. Flow cytometry analyses were performed to determine the alteration of cell cycle in different groups.

Results: HMGB2 was abnormally overexpressed in HCC. HMGB2 knockdown reduced malignant behaviors especially the proliferative potential and motility of HCC cells. The inhibition of HCC cells proliferation and mobility could be partially restored via treatment with recombinant vimentin protein. Our findings confirmed abnormal activation of HMGB2-ZEB1 vimentin axis facilitates HCC malignant proliferation and motility. The elevated HMGB2 expression in clinical samples was related to postoperative survival time of HCC patients. It indicated HMGB2 promotes the proliferation and motility potential of HCC via HMGB2-ZEB1-vimentin axis activation.

Conclusions: HMGB2 is up-regulated in HCC and affects the malignant transformation by modulating HMGB2-ZEB1-vimentin signaling pathway, which may provide a research basis for evaluating the disease progression and developing clinical treatment strategies of HCC.

Keywords: High mobility group box 2 (HMGB2); zinc finger E-box binding homeobox 1 (ZEB1); vimentin; malignant transformation; hepatocellular carcinoma (HCC)

Submitted May 25, 2023. Accepted for publication Sep 22, 2023. Published online Oct 24, 2023.

doi: 10.21037/jgo-23-447

View this article at: <https://dx.doi.org/10.21037/jgo-23-447>

Introduction

Primary liver cancer (PLC) is the fourth leading cause of cancer-related deaths worldwide, with hepatocellular carcinoma (HCC) accounting for the majority of PLC (1). HCC usually occurs in the background of cirrhosis caused by multiple risk factors, including chronic viral infection of hepatitis B virus (HBV) or hepatitis C virus (HCV), alcoholism, exposure to aflatoxin, non-alcoholic steatohepatitis (NASH), and drug-related liver injury (2). With the improvements of surgical skills and the assistance of comprehensive treatment strategies, such as radiotherapy, chemotherapy, and immunotherapy, provide the possibility of prolonging survival time for HCC patients. However, clinical analysis has revealed that the 5-year overall survival rate for patients with HCC remains less than 40% (3). This is explained by common postoperative metastasis and recurrence caused by intrahepatic or extrahepatic invasion and dissemination in a short time period (4,5). Therefore, further investigation of molecular mechanisms of occurrence and metastasis should identify key targets to regulate the survival and invasion of cancer cells, and therefore provide a new strategy for clinical therapy.

High mobility group box 2 (HMGB2), is a highly conserved nucleoprotein which belongs to high mobility group of protein family. Other identified family members include HMGB1 and HMGB3 (6).

HMGB2 is widely distributed in mammalian cells and participates in a variety of cellular functions, including erythrocyte production, sperm maturation, intracellular DNA transcription and replication (7). In normal physiological conditions, HMGB2 only exhibits high expression during embryogenesis. Its expression decreases with the maturation of individuals (8). In recent years, researchers have reported that HMGB2 is abnormally expressed in numerous human cancers, including HCC, colorectal cancer and glioma, which is closely related with the malignant biological phenotype and poor clinical prognosis of tumors (9-12). Up to now, the definitive role of HMGB2 in HCC is largely unknown and requires further investigation. Based on the existing research, we speculate that the abnormal expression of HMGB2 is involved in the malignant progression of HCC, but the specific mechanism needs to be further explored.

The purpose of our work is to explore the level of HMGB2 in clinical tissues and cells. Furthermore, its effects on the biological characteristics of HCC and related molecular mechanisms were evaluated. In summary, our study attempts to explore the possibility of HMGB2 as a novel biological target for clinical treatment of HCC. We present this article in accordance with the REMARK and MDAR reporting checklists (available at <https://jgo.amegroups.com/article/view/10.21037/jgo-23-447/rc>).

Methods

Ethics statement

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Collection and use of clinical tissues, relevant pathological and follow-up data were approved by Biomedical Ethics Committee of The Third Affiliated Hospital of Navy Military Medical University (No. EHBHKY2020-02-003; Shanghai, China). Written informed consent was received from each patient prior to the surgery.

Patients and specimens

HCC tissues (n=62) were obtained from patients who received radical local hepatectomy in The Third Affiliated Hospital of Navy Military Medical University from February 2013 to January 2014. All cases were confirmed diagnosis of primary HCC by imaging, serological diagnosis and histopathology. All patients did not receive any antineoplastic therapy, such as drug treatment, chemotherapy

Highlight box

Key findings

- High mobility group box 2 (HMGB2) has the potential as an oncogene which participates in malignant transformation of hepatocellular carcinoma (HCC) via HMGB2-zinc finger E-box binding homeobox 1-vimentin signaling pathway.

What is known and what is new?

- HMGB2 is abnormally expressed in human cancers and closely related with the malignant biological phenotype and poor clinical prognosis. However, the elaborated mechanism of its role in regulating HCC progression is still poorly understood. This study provides a novel molecular mechanism and a new strategy for adjuvant clinical treatment of HCC in the future.

What is the implication, and what should change now?

- This study suggested that HMGB2 may be a key regulatory factor in promoting the malignant progression of HCC. Targeted intervention of key proteins in its signaling pathway may become a novel approach for adjuvant therapy of HCC. The following research requires multi animal models and large cohorts clinical sample validation, as well as screening targeted drugs.

or radiotherapy before operation and were postoperatively followed up as previously described (13). In brief, the last follow-up was done in April 2019, with a median follow-up time of 41.0 months (range, 5.5–69.0 months). The clinicopathological characteristics of HCC patients were summarized in *Table 1*. The tissue samples obtained from surgical resection are used for tissue microarray (n=62, for immunohistochemical analysis) and RNA extraction [n=62, for quantitative real-time polymerase chain reaction (qRT-PCR) detection].

Cell culture

Human HCC cells included MHCC97H, Hep3B, LM3 and Huh7, and immortalized normal liver cell line THLE-2 were used for cell experiments (The Cell Bank of the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences). All cells were cultured as previously described (13). In brief, all the cells were cultured at 37 °C, 5% CO₂ and 95% humidity condition. All experimental cells involved in the study had been identified and confirmed by short tandem repeats (STRs) profiling and mycoplasma testing. HMGB2 small interfering RNAs (siRNAs) and scramble siRNA were artificially synthesized by RiboBio Co., Ltd. (siG000003148; Guangzhou, China). Detailed information of sequence is summarized in *Table S1*. siRNA transfection was optimized using Lipofectamine 3000 (catalog No. L3000001, Invitrogen; Thermo-Fisher Scientific, Inc., Carlsbad, CA, USA). Recombinant human vimentin protein was obtained from R&D Systems, Inc. (catalog no, 2105-VI-100; Minneapolis, MN, USA).

Cell viability, migration and invasion assays

Cell proliferation activity was evaluated by using cell counting kit-8 assay (CCK-8 assay; catalog No. C0038; Beyotime Institute of Biotechnology, Nantong, China). In short, cells were digested and resuspended as single cell suspension, and then counted and inoculated on 96-well cell culture plates with a density of 5,000 cells per well. The experimental group was divided into three groups: blank control group, scramble control group [transfected with scramble control by lipofectamine 3000 (catalog No. L3000001, Invitrogen; Thermo-Fisher Scientific, Inc.)] and HMGB2 siRNA group [transfected with HMGB2 siRNA by lipofectamine 3000 (catalog No. L3000001, Invitrogen; Thermo-Fisher Scientific, Inc.)]. After routine

cultivation for 24, 48 and 72 h, we detected each absorbance at 490 nm by using a spectrophotometer (Multiskan FC, Thermo-Fisher Scientific, Inc.). Cell migration & invasion experiments were carried out on transwell chambers with or without Matrigel-coating (catalog No. 354480, Corning Inc., NY, USA), as previously described (13). After 48 h of transfection, count the number of cells passing through the chambers in different experimental groups. Each experiment was repeated three times.

Immunohistochemistry

Immunohistochemical detection of HCC tissue microarray was conducted as described previously (13). The following antibodies were used: HMGB2 antibody (catalog No. ab133540; dilution: 1:100; Abcam, UK), zinc finger E-box binding homeobox 1 (ZEB1) antibody (catalog No. ab203829; dilution: 1:100; Abcam) and vimentin antibody (catalog No. ab8979; dilution: 1:200; Abcam). Staining evaluation was performed by three investigators. Immunoreactive evaluation was performed as previously described (13). For immunofluorescence detection, HCC cells were inoculated and cultured on cover slides in 6-well culture plates. Following treatment, cell fixation, membrane penetration and primary antibody incubation followed by the appropriate Alexa Fluor 647/Cy3/Alexa Fluor 488-labeled secondary antibodies (catalog nos. A0468, A0516 and A0428; Beyotime Institute of Biotechnology), and co-stained using 4',6-diamidino-2-phenylindole (DAPI) (catalog No. MBD0015; Sigma-Aldrich: Merck KGaA, St. Louis, USA).

Reverse transcription-quantitative PCR (RT-qPCR)

HCC tissues and cells were dissociated by using TRIzol reagent with the aim to extract the total RNA (catalog No. 15596026; Thermo-Fisher Scientific, Inc.). Reverse transcription and polymerase chain reactions (PCRs) were used to amplify the target genes (StepOnePlus, Applied Biosystems; Thermo-Fisher Scientific, Inc.). The reaction conditions were carried out as conventional procedures. Three replicates were performed with each cDNA sample. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was determined as control of target genes. The $2^{-\Delta\Delta C_t}$ system was used to analyze the results. The primer sequences of all detected target genes are summarized in *Table S2*.

Table 1 Correlation between HMGB2 expression and HCC clinicopathological features

Clinicopathologic features	No. of patient	HMGB2 expression		χ^2	P value
		Low (n=32)	High (n=30)		
Age (years)					
≤50	47	26	21	1.069	0.301
>50	15	6	9		
Gender					
Male	40	22	18	0.518	0.472
Female	22	10	12		
Etiology					
HBV infection	46	26	20	2.978	0.226
HCV infection	13	4	9		
Alcohol	3	2	1		
Background liver pathology					
Normal liver	3	2	1	0.495	0.781
Chronic hepatitis	20	11	9		
Liver cirrhosis	39	19	20		
Serum AFP (ng/mL)					
≤400	45	28	17	7.397	0.007
>400	17	4	13		
Tumor size (cm)					
≤5	41	29	12	17.717	<0.001
>5	21	3	18		
Intrahepatic metastasis					
Present	51	30	21	5.984	0.014
Absent	11	2	9		
Vascular invasion					
No	43	27	16	7.020	0.008
Yes	19	5	14		
Tumor encapsulation					
Yes	24	14	10	0.708	0.401
No	38	18	20		
Early recurrence					
No	33	21	12	4.084	0.043
Yes	29	11	18		
TNM stage					
I	35	19	16	0.230	0.632
II + III	27	13	14		

Chi-squared tests and Fisher's exact tests were employed to analyzed the clinical information. HMGB2, high mobility group box 2; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, α -fetoprotein; TNM, tumor, lymph node and metastasis.

Western blot analysis

Proteins were extracted from HCC samples and cells, then separated and visualized as previously described (13). The images were scanned and statistically analyzed using Image J V1.8.0 (National Institute of Health, Bethesda, MD, USA). The following antibodies were used: HMGB2 antibody (catalog No. ab133540; dilution: 1:1,000; Abcam), ZEB1 antibody (catalog No. ab203829; dilution: 1:1,000; Abcam), vimentin antibody (catalog No. ab8979; dilution: 1:2,000; Abcam), p53 antibody (catalog No. ab26; dilution: 1:1,000; Abcam) and vimentin antibody (catalog No. ab137872; dilution: 1:1,000; Abcam). GAPDH was used as internal control (catalog No. ab70699; dilution: 1:6,000; Abcam). The evaluation and statistical analysis of protein bands were carried out by employing Quantity One software (v4.6.6; Bio-Rad Laboratories, Inc., Hercules, USA).

Detection of cell cycle using flow cytometry

HCC cells were routinely cultured and transfected with different treatment groups. Cells were collected after 48 h of transfection. Cells were fixed with precooled 75% ethanol and incubated at 4 °C overnight. Then, they were stained with RNase-containing propidium iodide (PI) solution (catalog No. C1052; Beyotime Institute of Biotechnology) for 30 min in darkness after rinsing with phosphate buffered solution (PBS) for two times. Cell cycle changes were detected by BD flow cytometer (Accuri C6; Franklin Lakes, NJ, USA). The results were analyzed using FlowJo 10.0 software (Tree Star, Inc., Ashland, OR, USA). G0, G0 phase, quiescent period of the cell cycle; G1, G1 phase, the first stage of the cell cycle; G2, G2 phase, mitotic preparation period of the cell cycle; M, M phase, mitotic period of the cell cycle; S, S phase, DNA synthesis stage of the cell cycle.

Statistical analysis

All statistical analysis involved in the study were carried out by SPSS software (version 18.0; SPSS Inc., Chicago, IL, USA). Data is presented as median ± standard error. Student *t*-test was employed for comparison between groups. Chi-squared tests and Fisher's exact tests were employed to analyze the clinical information. Kaplan-Meier method was employed to further analyze prognosis and survival information of HCC patients. Multivariate Cox proportional hazards model was employed to further

analyze clinical data. There is a statistically significant difference when P value is less than 0.05.

Results

Nucleoprotein HMGB2 is overexpressed in HCC

HMGB2 protein expression level of 62 patients was evaluated in a paraffin-embedded tissue microarray. Our findings revealed that the protein expression of HMGB2 was elevated in 30 HCC patients, and predominantly located in the nucleus and cytoplasm (*Figure 1A*). In para-cancerous liver tissues the protein level of HMGB2 was lower. Similar results could also be found in mRNA expression detection (*Figure 1B*, $P < 0.001$). This result was also confirmed by western blot analysis with HCC cell lines. HMGB2 expression increased in HCC cells, particularly in MHCC97H and LM3 cells which are generally considered with high metastatic potential HCC cells (*Figure 2A*, $P < 0.05$), compared to normal liver cells (THLE2). Based on the high metastatic potential characteristics and high expression of HMGB2 in MHCC97H and LM3 cells, we ultimately chose these two cells for subsequent experiments. Our findings suggested ZEB1 and vimentin protein expression were higher in HCC samples accompanied with an increase of HMGB2 and mRNA and protein (*Figure 1A, 1B*, $P < 0.001$). ZEB1 is a transcription factor that promotes metastasis and stem cell characteristics (14). Epithelial mesenchymal transition (EMT) is considered a crucial mechanism of tumor metastasis, and vimentin is one of the most important protein markers related to metastasis in this process (15). These results suggested that HMGB2 was abnormally overexpressed in HCC tissue samples and cell lines. Furthermore, the protein expression of HMGB2, ZEB1 and vimentin were explored in early and advanced stage HCC tissues. It was observed that HMGB2, ZEB1 and vimentin were expressed at higher levels in advanced HCC samples (*Figure S1*), indicating that these proteins might be related with the malignant development of HCC. HMGB2, a nucleoprotein, can regulate various biological processes by interacting with transcription factors such as p53, p73, lymphoid enhancer-binding factor 1 (LEF1), and Runt-related transcription factor 2 (Runx2) (16-19). Nuclear transcription factor ZEB1 has been reported to facilitate tumor progression in inducing EMT of tumor cells. In addition, the protein level of ZEB1 is positively correlated with vimentin expression (20). Additionally, the present results revealed that HMGB2 could affect the

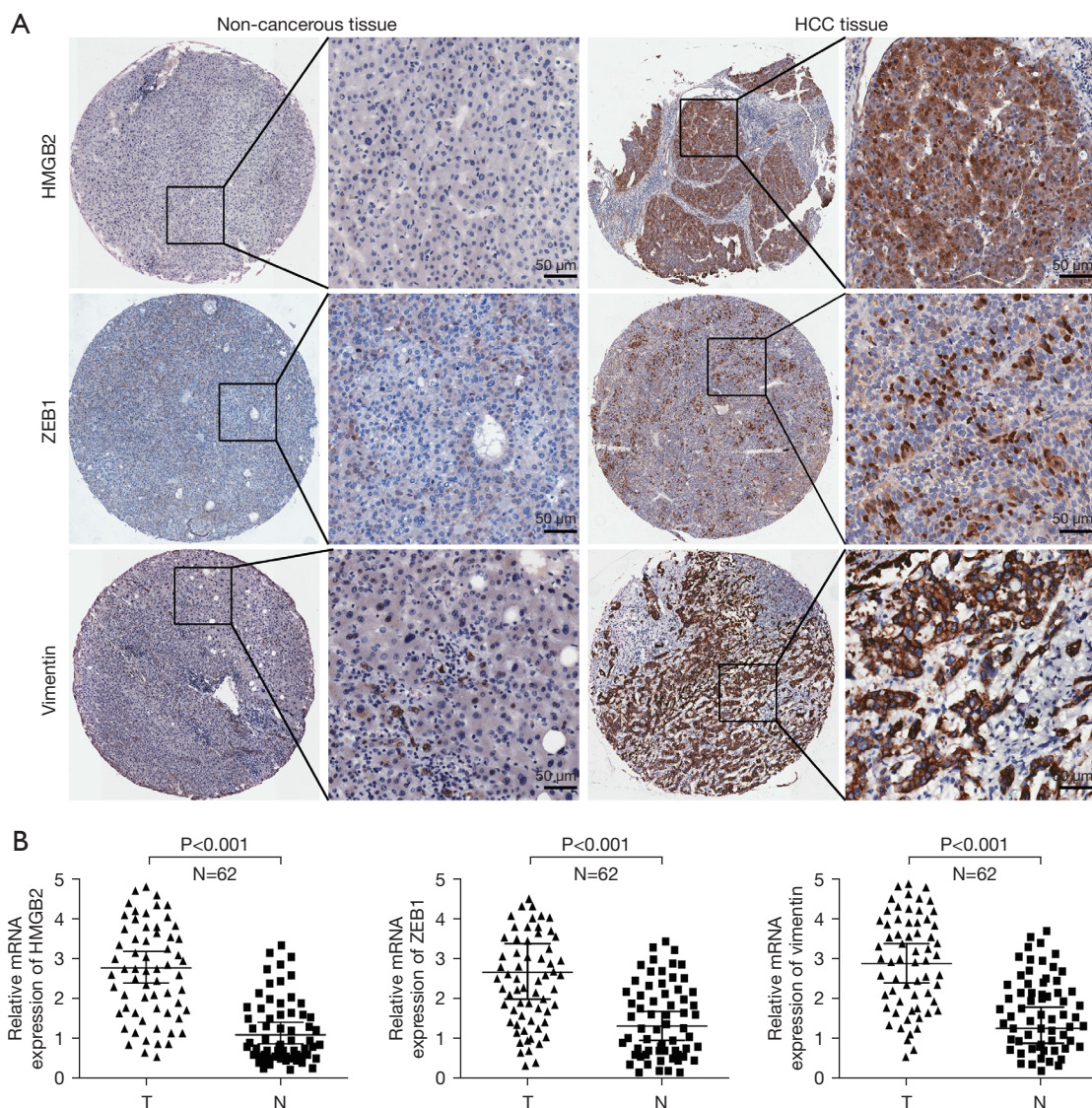


Figure 1 HMGB2 is highly expressed in hepatocellular carcinoma tissues. (A) Representative fields of HMGB2, ZEB1 and vimentin protein expression in HCC samples (n=62, scale bar =50 μm). DAB and hematoxylin staining methods were used to detect cytoplasmic and nuclear staining, respectively. (B) PCR was performed to evaluate HMGB2 mRNA in cancer and para-cancerous tissues (n=62). Student *t*-test was employed for comparison between groups. HMGB2, high mobility group box 2; HCC, hepatocellular carcinoma; ZEB1, zinc finger E-box binding homeobox 1; T, tumor tissue; N, non-tumor tissue; DAB, 3,3'-diaminobenzidine; PCR, polymerase chain reaction.

expression of the ZEB1/vimentin axis.

HMGB2 deprivation restricts the proliferation, migration and invasion ability of HCC cells in vitro

Firstly, the effect of HMGB2-knockdown in MHCC97H and LM3 HCC cells was assessed by western blotting. siRNA1 presented the strongest interference function

(Figure 2B, $P < 0.05$). The HCC cells transfected with HMGB2 siRNA had a weaker proliferative capacity compared to the control and scramble groups (Figure 3A, $P < 0.05$). The results of the transwell assay suggested that HMGB2 siRNA1 dramatically inhibited the migratory and invasive potential compared to control and scramble groups (Figure 3B, $P < 0.05$). Notably, the inhibition of proliferation and mobility potential of HCC cells *in vitro* could be

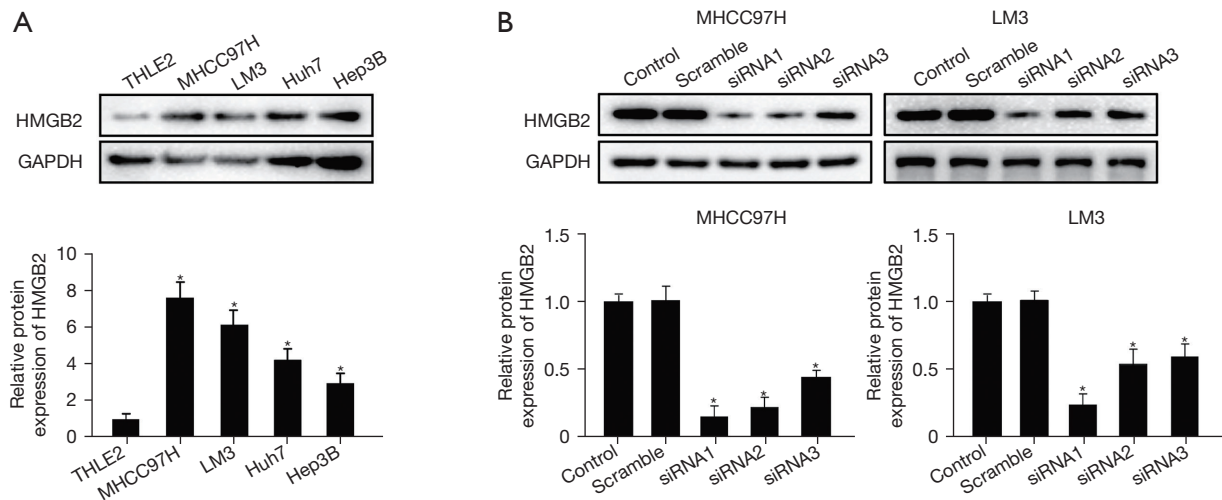


Figure 2 HMGB2 is highly expressed in HCC cells. (A) HMGB2 protein expression levels in HCC cells. (B) Detection of HMGB2 protein expression levels of MHCC97H and LM3 HCC cells in each treatment group. We repeated each experiment three times independently. Student *t*-test was employed for comparison between groups. *, $P < 0.05$ vs. control and scramble group. HMGB2, high mobility group box 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA; HCC, hepatocellular carcinoma.

partially restored by recombinant human vimentin protein (10 μ M) (Figure S2, $P < 0.05$). Surprisingly, HMGB2 showed no significant effect on the cell cycle (Figure S3). The above findings suggest that decreased expression of HMGB2 could restrict the malignant proliferation, migration, and invasion of HCC cells.

HMGB2 knockdown affects the malignant characteristics of HCC cells via regulating the ZEB1/vimentin axis

With the aim of evaluating the molecular mechanism of HMGB2 in HCC cells, the relative expression levels of ZEB1/vimentin axis-associated protein were evaluated by western blotting and immunochemical staining. Our present results demonstrated that HMGB2 knockdown in HCC cells decreased ZEB1 and vimentin expression (Figure 4A,4B). As a nucleoprotein, HMGB2 can regulate various biological processes by binding transcription factors, such as p53, p73, LEF1 and Runx2. In our present study, p53 protein level was elevated and LEF1 decreased following the inhibition of HMGB2 expression (Figure 4A,4B, $P < 0.05$). In addition, immunochemical staining demonstrated that ZEB1 and vimentin expression decreased following HMGB2 knockdown in HCC cells (Figure 4C). Overall, HMGB2 overexpression may activate the ZEB1/vimentin axis in HCC.

Correlations of HMGB2 in HCC with clinicopathological indicators

Differences between clinicopathological indicators of HCC subtypes with high or low HMGB2 expression are presented in Table 1. HMGB2-high HCC subtypes were significantly associated with serum α -fetoprotein (AFP) level ($P = 0.007$), tumor size ($P < 0.001$), intrahepatic metastasis ($P = 0.014$), vascular invasion ($P = 0.008$) and early recurrence ($P = 0.043$).

Based on the analysis of multivariate Cox's proportional hazard models, clinicopathologic features were selected to be further evaluated (Table 2). HMGB2 ($P = 0.038$), vascular invasion ($P = 0.005$) and early recurrence ($P = 0.001$) were independent factors for prognosis.

HCC subtypes with high HMGB2 expression predict a poor prognosis

As presented in Figure 5, HCC cases with elevated HMGB2 expression were associated with a lower overall survival rate ($P = 0.0006$) and disease-free survival rate ($P < 0.0001$) compared to HCC patients with decreased HMGB2 expression.

Discussion

HCC is currently the sixth most prevalent malignant cancer and the third most lethal type of tumor worldwide (21-23).

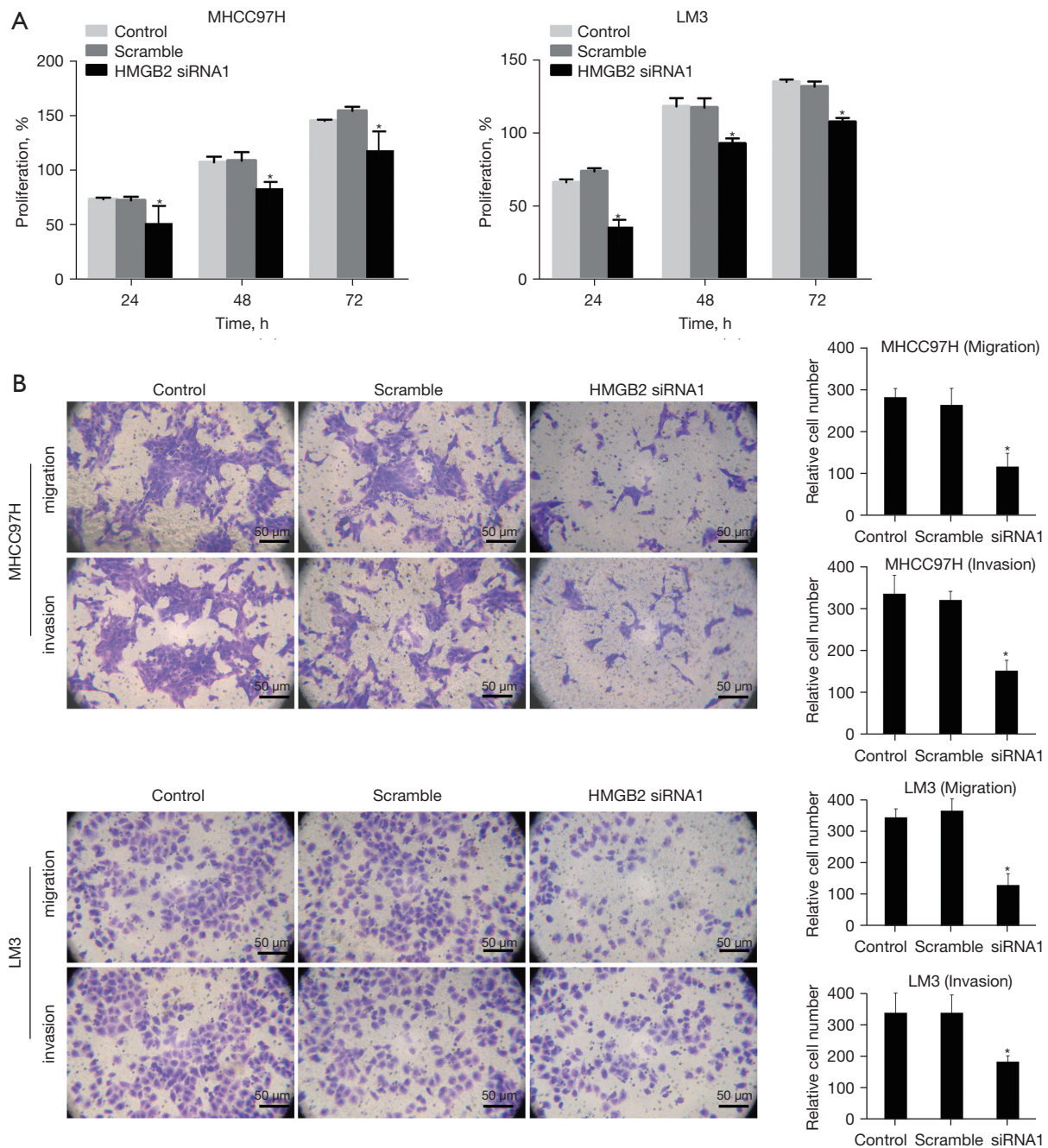


Figure 3 Deprivation of HMGB2 restrained the proliferation and motility of HCC. (A) HMGB2-knockdown effects on HCC cell proliferation were assessed by CCK-8 assay. (B) HMGB2-knockdown impacted on the migration and invasion ability of HCC cells were assessed by transwell chamber assays. Scale bar = 50 μ m. 1% crystal violet stain solution was used to label cells. We repeated each experiment three times independently. Student *t*-test was employed for comparison between groups. *, $P < 0.05$ vs. control and scramble group. HMGB2, high mobility group box 2; siRNA, small interfering RNA; HCC, hepatocellular carcinoma; CCK-8, cell counting kit-8.

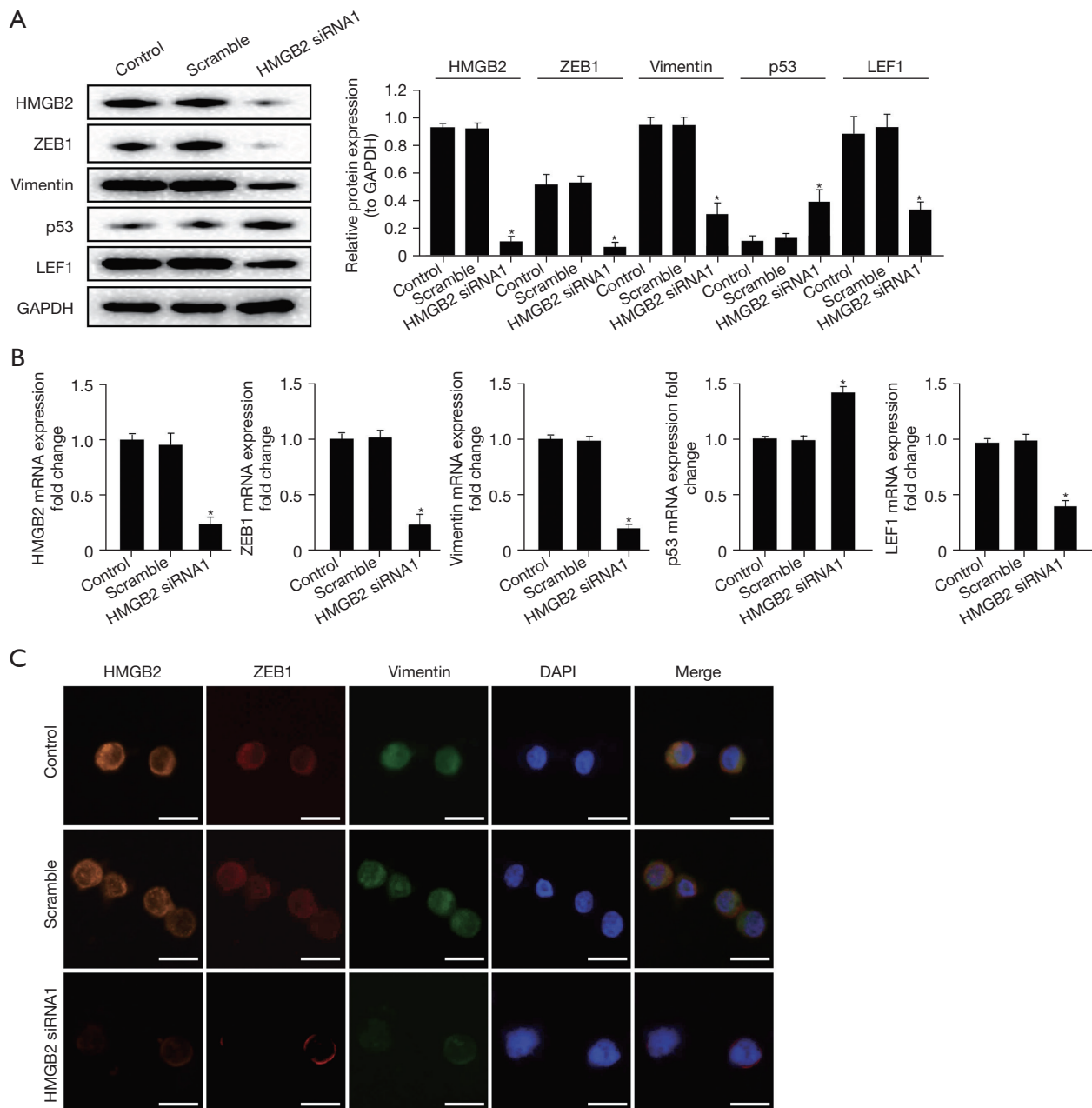


Figure 4 HMGB2 facilitates the proliferative and migratory potential of hepatocellular carcinoma cells by activating the HMGB2-ZEB1-vimentin axis. (A) HMGB2 knockdown significantly downregulated ZEB1, vimentin, p53 and LEF1 protein expression levels in MHCC97H cells. (B) PCR was employed to evaluate ZEB1, vimentin, p53 and LEF1 mRNA expression levels following HMGB2 knockdown in MHCC97H cells. *, $P < 0.05$ vs. control and scramble group. (C) Immunofluorescence staining with HMGB2, ZEB1 and vimentin antibodies, separately (scale bar = 20 μm). DAPI staining solution (blue), Alexa Fluor 647 (red), Cy3 (orange), and Alexa Fluor 488 (green)-labeled secondary antibodies were used for detection. We repeated each experiment three times independently. Student *t*-test was employed for comparison between groups. HMGB2, high mobility group box 2; ZEB1, zinc finger E-box binding homeobox 1; LEF1, lymphoid enhancer-binding factor-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA; DAPI, 4',6-diamidino-2-phenylindole; PCR, polymerase chain reaction.

Table 2 Multivariate Cox's proportional hazard models (n=62)

Term	Risk ratio	95% confidence interval	P value
Age	0.532	0.198–1.428	0.210
Gender	1.083	0.490–2.395	0.843
Etiology	1.446	0.854–2.449	0.170
Background liver pathology	2.040	0.760–5.475	0.157
Serum AFP	0.372	0.136–1.018	0.054
Tumor size	1.322	0.395–4.425	0.650
Intrahepatic metastasis	3.412	0.759–15.328	0.109
Vascular invasion	3.494	1.454–8.396	0.005
Tumor encapsulation	1.703	0.682–4.256	0.254
Early recurrence	4.408	1.897–10.238	0.001
TNM stage	1.950	0.876–4.339	0.102
HMGB2	2.674	1.058–6.763	0.038

Multivariate Cox proportional hazards model were employed to further analyze clinical data. AFP, α -fetoprotein; TNM, tumor, lymph node and metastasis; HMGB2, high mobility group box 2.

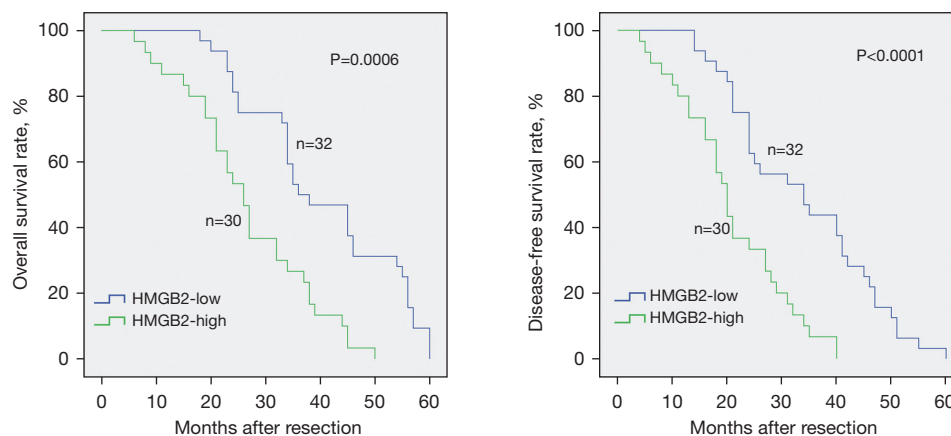


Figure 5 Elevated HMGB2 indicates poor prognosis in HCC patients. OS and DFS of HCC patients (n=62) were classified as HMGB2-low group (n=32) and HMGB2-high group (n=30) according to the protein expression of HMGB2. Elevated expression of HMGB2 was related to shorter OS ($P=0.0006$) and DFS ($P<0.0001$) rates. Kaplan-Meier method was employed to further analyze prognosis and survival information of HCC patients. HMGB2, high mobility group box 2; HCC, hepatocellular carcinoma; OS, overall survival; DFS, disease-free survival.

Early recurrence and metastasis postoperatively are the most impactful indicators affecting the long-term survival of HCC (24–26). Therefore, identifying novel predictors of recurrence and metastasis is essential for the clinical therapy of HCC.

HMGB2 is a crucial member of HMG protein

superfamily, which contains a DNA domain (HMG-box) (27). HMGB2, a nucleoprotein, can regulate various biological processes by interacting with transcription factors such as p53, p73, LEF1, and Runx2. (16–19). In addition, HMGB2 can regulate the development and differentiation of different cells. In recent years, accumulating studies (9–12)

have shown that abnormal elevation of HMGB2 can play an important role as a biomarker of tumor progression, metastasis, differentiation and it can also predict the prognosis of patients. However, up to now, the explicit function of HMGB2 in HCC is still a mystery.

Previous studies have demonstrated that HMGB2 is abnormally expressed in human cancers and contributes to the regulation and maintenance of malignant biological phenotype and clinical prognosis of patients (9-12). In this study, we further demonstrated that HMGB2 was overexpressed in HCC samples and cell lines, particularly in advanced HCC tissues compared to para-cancerous liver tissues. Further analysis between clinicopathological data and HMGB2 confirmed that the high expression of HMGB2 was closely related with serum AFP level, tumor size, intrahepatic metastasis, vascular invasion and early recurrence. Therefore, it is speculated that the abnormal overexpression of HMGB2 may participate in multiple stages of malignant progression of HCC.

HMGB2 siRNA was taken to eliminate the expression in MHCC97H and LM3 HCC cells. Our findings suggested that the proliferation and mobility potential of HCC cells were dramatically inhibited *in vitro*. Although the standard deviation may be caused by some deviation of cell count, the overall trend of the experimental results is consistent, repeatable and reliable. These results confirmed that HMGB2 participates in the regulation of malignant biological characteristics of HCC cells, such as proliferation, migration and invasion.

Mechanistically, we demonstrated that HMGB2 promotes malignant biological behaviors which are dependent on ZEB1-vimentin axis. Vimentin as an intermediate filament protein, is an important marker of the mesenchymal phenotype, which maintains the integrity of the cytoskeleton and promotes the motility of multiple tumor cells (28-30). Tumor cells often exhibit EMT phenotype during metastasis, and Vimentin promotes EMT by altering cell shape and motility characteristics. Proteomics related research reported that vimentin is a metastasis related factor of various malignant tumors, such as prostate cancer and breast cancer, which indicates that it may play an important role in tumor progression and become a potential biomarker of metastasis (31). ZEB1, a master regulator of the EMT, which regulates the expression of vimentin (32), has been reported to facilitate tumor invasion and metastasis by inducing EMT in various tumors. ZEB1 belongs to the E-box binding zinc finger

protein family and promotes downstream gene transcription by binding to E-box promoter elements (33). High expression of ZEB1 is also common in highly metastatic epithelial tumor cells (34). The present results showed that HMGB2, ZEB1 and vimentin were overexpressed in a considerable number of HCC tissue samples. Following HMGB2 knockdown by siRNA, ZEB1 and vimentin expression levels decreased simultaneously. Therefore, it was speculated that HMGB2 may bind to the nuclear transcription factor ZEB1 directly or indirectly, affecting the expression and role of vimentin, which impacts on the malignant features of HCC by activating the HMGB2-ZEB1-vimentin axis.

A particularly interesting phenomenon was that HMGB2 particularly highly expressed in advanced HCC samples. It indicated that HMGB2 participated in the malignant development of HCC which could be used as a novel monitoring and evaluation index. The signal transduction in the tumor microenvironment is complex, accompanied by abnormal activation of HMGB2 in the early stage of tumor occurrence. The body may neutralize its expression through some unknown mechanism to maintain the homeostasis. As the disease progresses, when HMGB2 protein accumulates in sufficient, it will break the balance and activate a series of proteins related to invasion and metastasis, ultimately leading to malignant transformation of tumor cells such as invasion and metastasis. Besides, the inhibition of HCC cell proliferation and mobility *in vitro* could be partially restored by recombinant human vimentin protein. However, the specific mode of interaction and regulation of the HMGB2-ZEB1-vimentin axis needs to be verified by subsequent experiments. In future work, ZEB1 recombinant protein alone or in combination with vimentin is worthy of further study on ZEB1/vimentin signal pathway. HMGB2 as a drug target is also worth paying more attention to investigate in the future. For example, analyze its protein structure through X-ray crystallography for drug design and localization, and finally match drugs with targets. Meanwhile, biochip technology can also be employed to obtain tens of thousands of target information simultaneously, and achieve rapid screening and drug optimization.

Finally, the clinical and prognostic data of HCC patients indicated that elevated HMGB2 could be an independent factor to predict prognosis and survival. It also confirmed the perspective that HMGB2 was involved in tumor progression. Taken together, these data indicate HMGB2

participates in malignant progression of HCC, which expected to become an attractive molecular target for HCC in the future. Our current understanding of HMGB2 in HCC is far from fully explaining its mechanism. Our study uncovered several key observations and mechanisms of action on animal models or larger cohorts of patients which need to be further explored in the future research.

Conclusions

HMGB2 participates in the malignant development of HCC and it is a previously unknown correlation with ZEB1-vimentin axis. We broaden the knowledge of HMGB2 action in HCC progression. Considering the universal role of HMGB2 in promoting tumor cell proliferation and metastasis, combining vimentin inhibitor or ZEB1 inhibitor might improve the anti-tumor effect as a widely applicable new approach in cancer therapy in the future.

Acknowledgments

We would like to acknowledge the helpful comments on this paper received from our reviewers and editors.

Funding: None.

Footnote

Reporting Checklist: The authors have completed the REMARK and MDAR reporting checklists. Available at <https://jgo.amegroups.com/article/view/10.21037/jgo-23-447/rc>

Data Sharing Statement: Available at <https://jgo.amegroups.com/article/view/10.21037/jgo-23-447/dss>

Peer Review File: Available at <https://jgo.amegroups.com/article/view/10.21037/jgo-23-447/prf>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://jgo.amegroups.com/article/view/10.21037/jgo-23-447/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was

conducted in accordance with the Declaration of Helsinki (as revised in 2013). Collection and use of clinical tissues, relevant pathological and follow-up data were approved by Biomedical Ethics Committee of The Third Affiliated Hospital of Navy Military Medical University (No. EHBHXY2020-02-003; Shanghai, China). Written informed consent was received from each patient prior to the surgery.

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Cite this article as: Lu K, Zhao T, Yang L, Liu Y, Ruan X, Cui L, Zhang Y. HMGB2 upregulation promotes the progression of hepatocellular carcinoma cells through the activation of ZEB1/vimentin axis. *J Gastrointest Oncol* 2023;14(5):2178-2191. doi: 10.21037/jgo-23-447

Table S1 The target sequences of siRNA-HMGB2

Term	Sequences
si-HMGB2-scramble	Forward: UUCUCCGAACGUGUCACGUdTdT Reverse: ACGUGACACGUUCGGAGAAdTdT
si-HMGB2-1	Forward: GCGUUAGCGAGAAACCAGUTT Reverse: ACUGGUUUUCUCGCUAACGCTT
si-HMGB2-2	Forward: GAAAUCGCCCAAAGAUCATT Reverse: UGAUCUUUGGGCGAUUUUCTT
si-HMGB2-3	Forward: GGAAAAGUCCAAGUUUGAATT Reverse: UUCAAAAUUGGACUUUUUCTT

siRNA, small interfering RNA; HMGB2, high mobility group box 2.

Table S2 The information of primer sequences

Genes	Sequences
<i>GAPDH</i>	Forward: GTCTCCTCTGACTTCAACAGCG Reverse: ACCACCCTGTTGCTGTAGCCAA
<i>HMGB2</i>	Forward: TGACAAAGCTCGTATGACAGG Reverse: GGAAGAAGGCAGATGGTGGC
<i>ZEB1</i>	Forward: ACCCTTGAAAGTGATCCAGC Reverse: CATTCCATTTTCTGTCTTCCGC
<i>vimentin</i>	Forward: AAATGGCTCGTCACCTTCGT Reverse: TTGCGCTCCTGAAAACTGC
<i>p53</i>	Forward: CCTCAGCATCTTATCCGAGTGG Reverse: TGGATGGTGGTACAGTCAGAGC
<i>LEF1</i>	Forward: GACGAGATGATCCCCTTCAA Reverse: CGGGATGATTCAGACTCGT

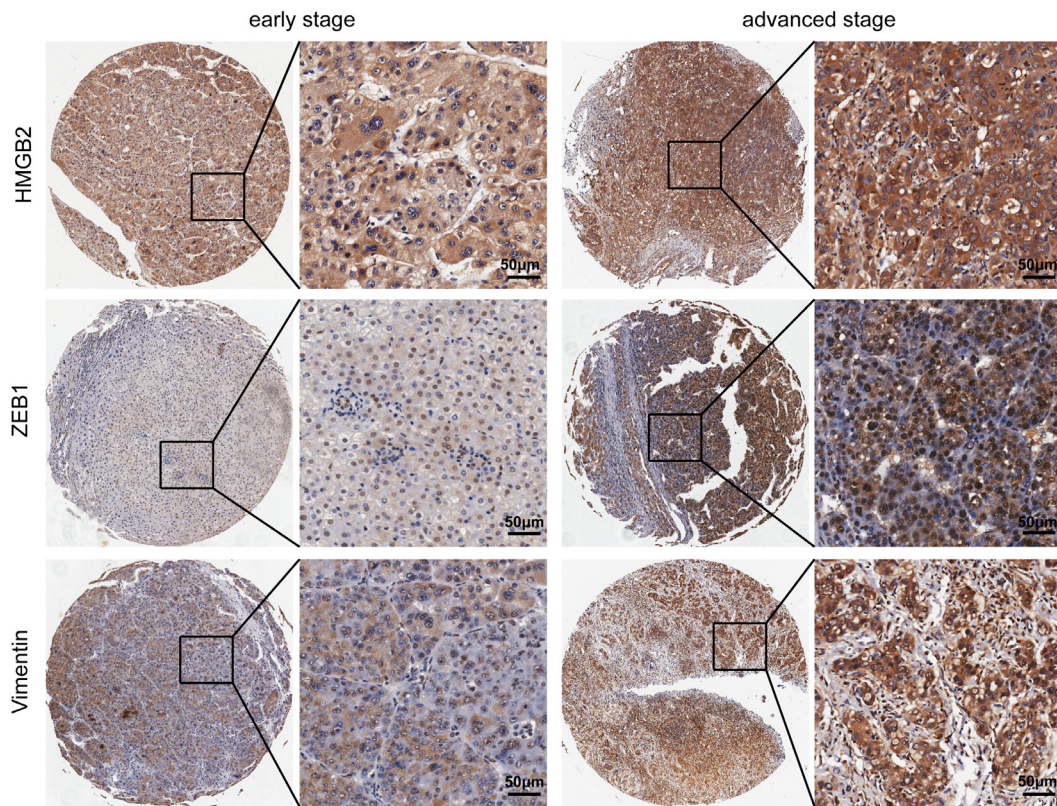


Figure S1 HMGB2 is highly expressed in advanced HCC tissues. Representative fields demonstrating HMGB2, ZEB1 and vimentin protein level in early stage and advanced HCC samples (scale bar =50 µm, n=5). DAB and hematoxylin staining methods were used to detect cytoplasmic and nuclear staining, respectively. HMGB2, high mobility group box 2; ZEB1, zinc finger E-box binding homeobox 1; HCC, hepatocellular carcinoma; DAB, 3,3'-diaminobenzidine.

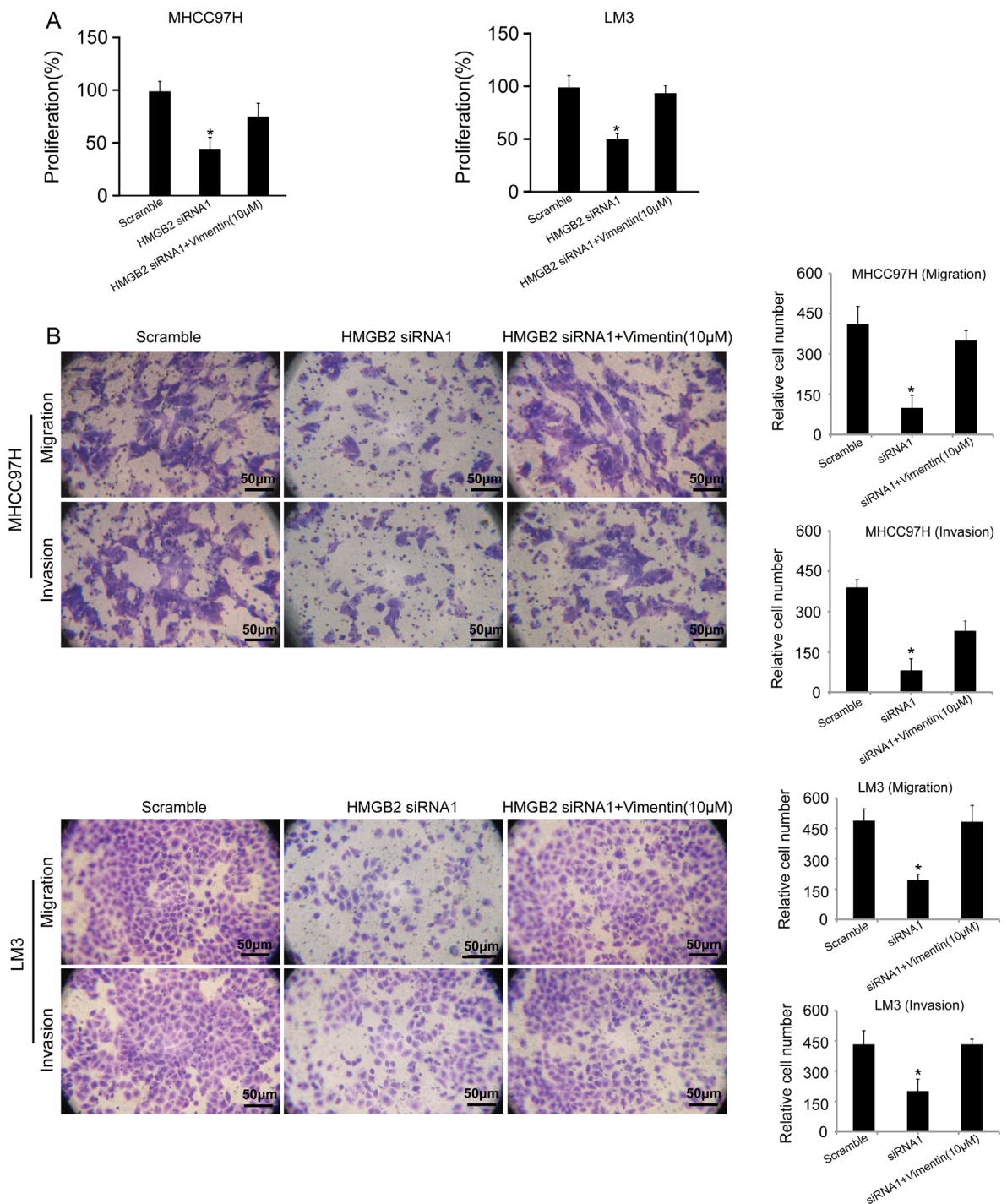


Figure S2 Recombinant human vimentin protein could partially restore the effect of HMGB2. (A) The effects of co-culture with HMGB2 siRNA and recombinant human vimentin protein on HCC cell proliferation were assessed by using CCK-8 assay. (B) The results of co-culture with HMGB2 siRNA and recombinant human vimentin protein on motility potential of HCC were evaluated by transwell. Scale bar =50 µm. 1% crystal violet stain solution was used to label cells. We repeated each experiment three times independently. Student *t*-test was employed for comparison between groups. *, $P < 0.05$ vs. scramble group and co-culture group. HMGB2, high mobility group box 2; siRNA, small interfering RNA; HCC, hepatocellular carcinoma; CCK-8, cell counting kit-8.

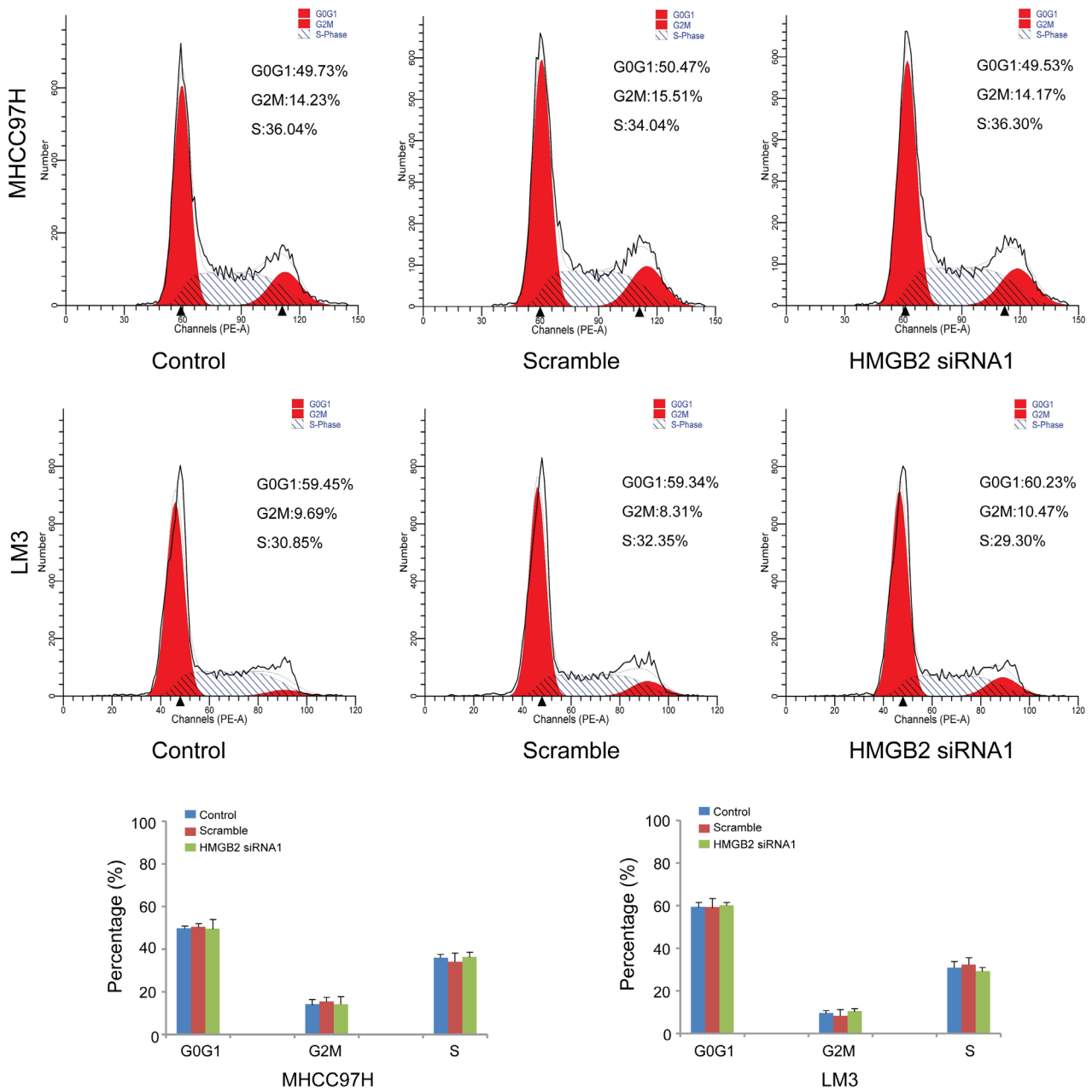


Figure S3 HMGB2 do not affect the cell cycle progression of HCC cells. Cells were collected after transfection with HMGB2 siRNA1 for 48 h. Cell cycle changes were detected by flow cytometry. G0, G0 phase, quiescent period of the cell cycle; G1, G1 phase, the first stage of the cell cycle; G2, G2 phase, mitotic preparation period of the cell cycle; M, M phase, mitotic period of the cell cycle; S, S phase, DNA synthesis stage of the cell cycle. We repeated each experiment three times independently. Student *t*-test was employed for comparison between groups. HMGB2, high mobility group box 2; siRNA, small interfering RNA; HCC, hepatocellular carcinoma.