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***Basic Study***

**Abnormal expression of *HMGB-3* significantly associated with malignant transformation of hepatocytes**

Zheng WJ *et al*. HMGB-3 in HCC

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**Abstract**

***AIM***

To explore the relationship between dynamic expression of high mobility group box-3 (HMGB-3) and malignant transformation of hepatocytes.

***METHODS***

Expressions of HMGB family were observed in rat hepatocarcinogenesis models induced with 2-acetylaminofluorene. Alterations of HMGB3 were analyzed at mRNA levels by RT-qPCR or at protein level by immunohistochemistry or Western blotting. HMGB3 in human liver cancer tissues were evaluated using bioinformatics databases from GEO, TCGA, and Oncomine, and HMGB3 gene transcription was intervened by specific HMGB3-shRNA to investigate on effects of proliferation and cell cycle of HepG2 cell lines *in vitro* or xenograft growth *in vivo*.

***RESULTS***

Elevated HMGB3 levels were first reported in hepatocarcinogenesis, with increasing expression from normal liver to cancer. Bioinformatic databases showed that HMGB3 expression in hepatocellular carcinoma tissues was significantly higher than that in normal liver tissues. Higher HMGB3 expressions were discovered in liver cancer cells compared with LO2 cells *in vitro*. According to gene set enrichment analysis, HMGB3 mRNA levels were correlated with cell cycle and DNA replication pathways. Knockdown HMGB3 by specific shRNA significantly inhibited proliferation of HepG2 cells with cell cycle arrest, downregulating DNA replication related genes (cyclin B1, FEN1, and PCNA) at mRNA or protein level. Furthermore, silencing HMGB3 significantly inhibited xenograft tumor growth with Ki67 reduction *in vivo*.

***CONCLUSION***

HMGB3 involved in malignant transformation of hepatocytes should be a useful biomarker for diagnosis and potential target therapy of liver cancer.

**Key words:** Liver cancer; *HMGB-3*; hepatocarcinogenesis; proliferation; tumor growth

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**Core tip:** high mobility group box (HMGB) family was correlated with hepatocellular carcinoma (HCC) development and progression. This current study examined the effects of HMGB3 on HCCboth *in vitro* and *in vivo*. Overexpression of HMGB3 was observed in hepatic malignant transformation and HCC tissues of Bioinformatic databases. Knockdown of HMGB3 significantly inhibited proliferation, cell cycle, and tumor growth of HCC cells, providing a novel insight for HCC research.

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**INTRODUCTION**

Hepatocellular carcinoma (HCC) is one of the most common and highly aggressive cancers worldwide, with the third most common cause of cancer death[1]. Various factors can cause HCC, including cirrhosis, infections with Hepatitis B virus (HBV) or HCV, nonalcoholic fatty liver disease (NFALD), diabetes, aﬂatoxin B1, tobacco, and excessive alcohol consumption. Among them, chronic infections with HBV and HCV account for more than 60% of total HCC cases[2,3]. In past decades, although obvious improvement has been observed in therapeutic approaches, the prognosis of HCC remains poor because of aggressive invasiveness, frequent metastasis and multi drug resistance (MDR). Given that multiple genes and signaling pathways play crucial roles in the occurrence and development of HCC, target therapy is a promising approach for HCC treatment[4]. Sorafenib, a multi-kinase inhibitor, has been approved as first-line treatment for advanced HCC by FDA. However, the overall effects are partially unsatisfying due to its low responsive rate and high frequency of adverse events[5,6]. Thus, it is of great importance to identify novel biomarkers for early diagnosis and potential targets against progression of HCC.

High mobility group (HMG)-box (HMGB) family belongs to HMG protein superfamily[7] (HMGA[8], HMGB[9], and HMGN[10]) and consists of 4 members (HMGB1, HMGB2, HMGB3, and HMGB4) with similar physiology and pathology features. It encodes proteins containing one or more DNA-binding motifs and participates in multiple cellular processes including cell differentiation, migration, and lots of inflammatory-related activities. HMGB family plays a complex role in carcinogenesis due to its diverse tumorigenic bioactivities in tumors. [HBV genome activates through functionally binding to HMG protein](https://www.ncbi.nlm.nih.gov/pubmed/11684893)[11]; mitochondrial biogenesis mediated by hypoxia promotes HCC growth through interaction between HMGB1 and Toll-like receptor 9[12]; HMGB1 secretion could be stimulated by HBX protein and subsequently enhance HCC metastasis[13]. HMGB1 signaling is also regulated by specific long noncoding RNA[14] or microRNA[15] showing pro- or anti- effects on invasion and metastasis of HCC[16]. Moreover, overexpression of HMGB2 is associated with aggressiveness and prognosis of HCC[17]. However, until now, there is rather less known about the HMGB3 or HMGB4 expression with HCC progression.

HMGB3 is a multifunctional protein with various roles in different cellular compartments and mainly distributed in cellular components of nucleus, chromosome, and cytoplasm. It contributes to the balance between self-renewal and differentiation of hematopoietic stem cells, and enhances DNA flexibility to activate gene promoters[18]. Recently, abnormal HMGB3 has been characterized as pro-carcinogenesis by promoting tumor growth, proliferation, invasion and metastasis in several tumors including gastric[19], lung[20], esophageal[21], breast[22], colorectal[23], and urinary bladder[24]. However, the current knowledge concerning the positive and negative effects of HMGB3 on HCC development is not explicit. The aims of this study were to investigate the dynamic HMGB3 expression in hepatocarcinogenesis, bioinformatics databases, HCC cell lines, and xenograft model, and to validate HMGB3 as a diagnostic marker or novel target gene for HCC.

**MATERIALS AND METHODS**

***Rat Hepatocarcinogenesis Model***

Total 40 Sprague-Dawley rats (4-6-wk-old) were provided by the Experimental Animal Center of Nantong University. Feeding condition included clean environment, 12-h light/dark cycle, and 55% humidity according to the previously described[25]. The rats of control group (*n* = 10) were fed normal diet, and the hepatocarcinogenesis group (*n* = 30) was fed diet with 0.05% 2-acetylaminofluorene (2-AAF, Sigma, United States). The rats were checked for status and sacrificed at different time. Rat livers were used for pathology, RNA extraction, and quantitative analysis of HMGB family expression. Following the determination of morphological changes of rat livers and hematoxylin and eosin (H&E) staining, the hepatocarcinogenesis group were divided into three sub-groups as degeneration (*n* = 6), precancerosis (*n* = 6), and HCC (*n* = 6), respectively. All procedures *in vivo* were performed according to the guidelines of Animal Care and Use Committee of Nantong University, China.

***Cell culture and transfection***

Human HCC cells HepG2, SMMC7721, HCCLM3, Huh7, BEL7404 and normal hepatocyte L02 were purchased from Cell bank of Chinese Academy of Science (Shanghai, China). HepG2, Huh7, and L02 HCCLM3 or SMMC7721, and BEL7404 were maintained in Dulbecco modified Eagle medium or RPMI1640 medium supplemented with 10% fetal bovine serum and antibiotics at 37℃ in a humidified incubator with 5% CO2. 3 candidate shRNAs were designed by Genema (Shanghai, China). Cell transfection was conducted according to manufacturer’s instructions. Briefly, once cells reached 80% confluence, plasmids were gently transfected into cells using transfection regent kit. After incubation for 12 h, cells were treated with fresh complete medium. The transfection efficiency was observed using fluore- scence microscope in 24 h. shRNA sequences were as follows: shRNA-1, 5’-GGAAAGTTTGATGGTGCAAAG-3’; shRNA-2, 5’-CGATCATATTGTAGT CTCTCA-3’; shRNA-3, 5’-CCTCCCTATAAATGTGGTAGC-3’; and NC- shRNA, 5’-GGAAGACGATGTCC GG G AAAG-3’.

***Histopathological Examination***

Sections of the formalin-fixed paraffin-embedded (FFPE) tissues were deparaffinized in xylene and rehydrated with a series of graded ethanol. Subsequently, following stained in hematoxylin solution for 15min, sections were counterstained in eosin solution using the Hematoxylin and Eosin (H&E) Staining Kit (Solarbio, China) according to the manufacturer’s instructions. Then samples were viewed under a light microscope.

***Bioinformatics Analysis***

In order to analyze the mRNA expression of HMGB3 in more HCC samples, gene expression profiling data from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (GSE-14520, GSE-5364, GSE-77314, and GSE-50579, USA), The Cancer Genome Atlas (TCGA) database, and Oncomine database (USA) were incorporated in this study. All data extracted from bioinformatics databases were presented as log2 value.

***Gene set enrichment analysis (GSEA)***

Gene set enrichment analysis (GSEA v2.2) was performed to discover the differences of biological process and signaling pathways in transcript levels between high and low HMGB3 expression in GSE-14520 and TCGA. Gene sets were obtained from the Molecular Signatures Database (MSigDB). Enrichment score (ES) were calculated to estimate genes from pre-defined gene set. The positive enrichment score indicated that the gene set was considered up-regulated while negative score meant down-regulated.  The number of permutations was set to 1000, and *P* <0.05 was considered significantly enriched.

***MTT assay***

Cell proliferation was detected with MTT assays. HepG2 cells with transfection of shRNA-1 and NC-shRNA were seeded in the 96-well plate at concentration of 3000 cells/well. From then, MTT solution (0.5mg/ml) was added to indicate wells at 1st ,2nd, 3rd, and 4th day. Following incubation for 4 hours, plates were added with DMSO solution. Then, the absorbance was detected in at the wavelength of 490 nm.

***Cell cycle***

Cells were collected by trypsin and fixed in 70% methanol for 30 min. Then cells were resuspended in PBS containing 50 µg/mL propidium iodide (Invitrogen, United States) for 1 h at room temperature. After that, samples were analyzed by a flow cytometer (BD Biosciences, United States). Percentage of each cycle phase was calculated by Modfit software.

***Immunohistochemistry***

Immunohistochemical analysis was performed as previously described[25]. In brief, tissue samples were fixed with formalin, embedded in paraffin, and cut into 4 μm-thick sections. Following incubation at 70 °C for 1 h, slides were deparaffinized in xylene, rehydrated with gradient ethanol. Then antigen retrieval was conducted using EDTA solution at pH 8.0. After blocked for 1 hour, slides were incubated with primary antibody overnight at 4℃ and further with secondary antibody for 2 hours at room temperature. After that, slides were visualized by DAB, and counterstained by hematoxylin.

***Western blotting***

Total protein was extracted form cell lysates using RIPA solution according to the manufacturers’ instructions and separated by 10% SDS-PAGE. Then the samples were transferred onto PVDF membranes. After blocking with PBS with 5% BSA, the membranes were incubated with primary antibodies in 1000 folds dilution (HMGB3, R&D, United States; Cyclin B1, FEN1, and PCNA, Abcam, United States; GAPDH, CST, United States) at 4℃ overnight. Following incubated with secondary antibody and washed by TBST, the samples were detected using ECL detection system (Bia-Rad, United States).

***Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR)***

Total RNA was extracted from tissues using Trizol (Invitrogen) according to the manufacturer’s instructions. cDNA was synthesized by using reverse transcription kit (Invitrogen, CA). Quantitative polymerase chain reaction (qPCR) was conducted by using SYBR Premix Ex Taq kit (Takara, Japan) according to the manufacturer’s instructions. The relative mRNA expressions (normalized to GAPDH) were assessed using the 2−ΔΔCt (ΔΔCt=ΔCt[target gene]−ΔCt[GAPDH]) analysis method. The primers in this study were presented inTable 1.

***Xenograft assay***

Male BALB/c nude mice (4-6 weeks old) were subcutaneously injected with 2 × 106 HepG2 cells transfected of shRNA-1 or NC-shRNA. Tumor size were measured every 4 d and calculated according to the formula (Volume= length × width2 × 1/2). Mice were sacrificed at the 30th day after injection. Tumors were weighed and fixed for further Immunohistochemistry of HMGB3 and Ki67 (1:50, Abcam, United States). All procedures were approved by Animal care committee of Nantong University.

***Statistics***

The data in this study are presented as means ± standard deviation (SD) of at least three experiments. Comparisons between groups were performed using Two-tailed Student’s *t*-test. *P* values less than 0.05 were considered statistically significant.

**RESULTS**

***Up-regulating Expression of HMGB3 in*** ***Hepatocarcinogenesis***

Construction of 2-AAF induced rat hepatocarcinogenesis model is shown in Figure 1. According to the morphological alteration and H&E staining, rats were divided into 4 groups: normal, degeneration, precancerous, and cancerous group. The degeneration group was characterized as the granule-like degeneration in the cytoplasm; the precancerosis group was characterized as dense nuclear chromatin and high ratio of nucleus to cytoplasm; the HCC group showed denser nuclear chromatin, upper ratio of nucleus to cytoplasm, and disappeared hepatic structure (Figure 1A). Then expressions of HMGB family (HMGB1, HMGB2, HMGB3, and HMGB4) were detected in liver tissues of each group above by RT-qPCR. As shown in Figure 1B-E, no statistically significant changes of HMGB1 (Figure 1B) or HMGB2 (Figure 1C) was found among groups above. Notably, hepatic HMGB3 expression had a significant increasing tendency during the transformation from normal hepatocytes to HCC (Figure 1D). However, HMGB4 expression trend was contrary to HMGB3, which is down-regulated in the HCC formation (Figure 1E). Further, dynamical up-regulating expressions of hepatic HMGB3 at protein level from normal to cancerous group (Figure 1F) were confirmed by the IHC staining with anti-HMGB3 antibody.

***Validation of HMGB3 mRNA by Bioinformatics Databases***

To further verify the HMGB3 expression in human HCC tissues, the data analysis of total normal livers (*n* = 359) and HCC (*n* = 765) tissues based on several bioinformatics databases (GEO, TCGA, and Oncomine) are shown in Figure 2. Compared with normal livers, HMGB3 had higher expression in HCC tissues according to the GSE14520 (fold change = 1.896, *t* = 11.270, *P* < 0.001, Figure 2A), GSE5364 (fold change = 1.720, *t* = 4.161, *P* = 0.002, Figure 2B), GSE77314 (fold change = 2.204, *t* = 4.473, *P* < 0.001, Figure 2C), and TCGA database (fold change = 1.709, *t* = 9.125, *P* < 0.001, Figure 2D). Meanwhile, GSE50597 presented up-regulated HMGB3 expression at advance stage of HCC in comparison with that at early stage (fold change = 2.054, *t* = 3.046, *P* = 0.012, Figure 2E). Besides, Oncomine database elucidated that higher HMGB3 expression was detected in HCC tissues rather than liver cancer precursor (fold change = 1.469, *t* = 2.948, *P* = 0.005) or normal livers (fold change = 1.795, *t* = 3.380, *P* = 0.003, Figure 2F). Given the observation above, the data suggested that the up-regulation of liver HMGB3 mRNA expression might be related to HCC progression.

***Knockdown of HMGB3 in HCC cell lines***

To further determine the role of HMGB3 in HCC progression, HMGB3 expression was detected and silenced in HCC cell lines (Figure 3). In contrast to normal hepatocyte L02, HMGB3 was overexpressed in HCC cell lines Huh7, HepG2, HCCLM3, SMMC7721, and BEL7404 (Figure 3A and B). Further, HepG2, with the highest expression of HMGB3, was chosen to conduct RNAi using three specific shRNAs with GFP labeling (Figure 3C). Compared with Control and NC-shRNA group, HMGB3 expression was significantly (*P* < 0.001) downregulated in shRNA-1 transfected group in mRNA (Figure 3D) and protein levels (Figure 3E and F).

***Silencing HMGB3 inhibited proliferation and regulated cell cycle of HCC cells***

The effects of silencing HMGB3 on HCC cells are presented in Figure 4. Gene set enrichment analysis was performed to sort the pathways enriched in distinct phenotype labels according to HMGB3 levels. In both of GSE14520 and TCGA, high expression of HMGB3 was correlated with cell cycle and DNA replication (Figure 4A). Thus, the proliferation activity and cell cycle were discovered in HMGB3-knockdown HepG2 cells. As shown in Figure 4B, knockdown of HMGB3 using shRNA-1 significantly inhibited the proliferation of HepG2 cells. Furthermore, silencing HMGB3 could lead to an obvious arrest of G1 phase in HepG2 cells (Figure 4C and D). In addition, consistent with the results of GSEA, HMGB3 knockdown also inhibited the expression of cell cycle and DNA replication related genes Cyclin B1, proliferating cell nuclear antigen (PCNA) and flap structure-specific endonuclease 1 (FEN1), indicating that HMGB3 might promote the proliferation of HCC cells by regulating cell cycle and DNA replication pathway (Figure 4E-G).

***Silencing HMGB3 inhibited tumor growth***

The effects of silencing *HMGB3* on tumor growth are shown in Figure 5. Compared with the NC-shRNA group, obviously smaller tumor volume and weight (*P* < 0.001) was observed in the shRNA-1 group at 30th day after subcutaneous injection (Figure 5A and C). In addition, silencing HMGB3 by shRNA-1 significantly impeded the growth of xenograft tumor according to the growth curves (Figure 5B). Furthermore, IHC results showed that Ki67 expression in tumor tissues of the shRNA-1 group was significantly lower than that of the NC-shRNA group, indicating that HMGB3 might contribute to the proliferation of HCC cells *in vivo* (Figure 5D).

**DISCUSSION**

HMGB family has been recognized as an important regulator in tumor progression[18]. Although HMGB family with various physiological and pathological functions was previously associated with liver cancer, neither HMGB1 nor HMGB2 was reported to exhibit dynamic expression in tumorigenesis[26]. In this study, the rat hepatocarcinogenesis model was conducted to analyze the expression characteristics from HMGB1 to HMGB4. No statistical differences of HMGB1 or HMGB2 among different groups were found during the dynamic alterations from normal rat hepatocytes to cancer formation. However, HMGB3 or HMGB4 expression presented up-regulated or down-regulated trend during the malignant transformation *in vivo*, respectively. To further verify the HMGB3 expression in HCC, several HCC-related bioinformatics databases were assessed. Interestingly, consistent with the results of HCC model, analyses of normalized log2 transformed microarray expression data sets clearly confirmed the significant up-regulating HMGB3 mRNA level in human HCC tissues and especially in advanced HCC tissues, indicating that HMGB3 might be an oncogenic protein involved in the malignant transformation of hepatocytes.

HMGB proteins can assist in either activating or repressing transcription[27]. In adult vertebrates, HMGB1 is found in all cell types, whereas HMGB3 mRNA was reported to be absent in most adult tissues[7,28]. Indeed, overexpression of HMGB3 has been discovered in several cancer types and correlated with clinical features of cancer patients, including advanced tumor-node-metastasis (TNM) stage, serosal invasion, and overall survival[23,28]. The rat hepatocarcinogenesis model also indicated the potential role of HMGB3 in HCC progression. However, the expression features of HMGB3 and its roles in HCC are still unclear. Thus, the current study further investigated HMGB3 expression in HCC cell lines. Interestingly, overexpression of HMGB3 was observed in HCC cells rather than normal hepatocytes, which was consistent with hepatocarcinogenesis model and bioinformatic analysis.

HMGB3 has been reported to play crucial roles in tumor progression by contributing to malignant behaviors and regulating oncogenic pathways. For instance, HMGB3 could enhance the migration and growth of gastric cancer cells *via* activation of Wnt pathway[23]; it also could increase the proliferation and invasion of breast cancer cells as a target gene of miRNA-205[29]. However, for HCC, the malignant behaviors and mechanism mediated by HMGB3 still remain unclear. Thus, bioinformatic analysis was conducted to explore the underlying roles. Notably, GSEA based on GEO database and TCGA jointly indicated that overexpression of HMGB3 might associate with cell cycle and DNA replication pathway. As expected, corresponding *in vitro* studies showed that silencing HMGB3 could significantly inhibit the proliferation and induce cell cycle arrest in HCC cells. To further confirm potential mechanism responsible for the anti-proliferation effects, we explored expression of Cyclin B1, PCNA, and FEN1, which were cell cycle and DNA replication related genes highly enriched in high-HMGB3-mediated pathways in GSEA. Consistent with prediction of GSEA, knockdown of HMGB3 obviously downregulated the three genes in mRNA and protein level, which have been reported to promote the tumor progression[30-32]. Collectively, HMGB3 might promote proliferation of HCC cells by regulating cell cycle and DNA replication pathways.

Although HMGB3 has been correlated with proliferation, chemoresistance, migration of cancer cells in previous studies[23,33,34], as far as we knew, there was no xenograft assays to evaluate HMGB3 as a regulator of tumor growth *in vivo*. Given the interesting results from rat model and *in vitro* study, our current study further evaluated HMGB3 as an important regulator of tumor growth *in vivo*. Remarkedly, xenograft tumors derived from HMGB3-silenced HCC cells grew slower than the control tumors, with obvious reduction in proliferation marker Ki67. It suggested that HMGB3 might contribute to the tumor growth *in vivo* via regulating proliferation of HCC cells.

In conclusion, to the best of our knowledge, this is the first report to investigate HMGB3 expression and indicate that it may be a novel diagnostic marker or therapeutic target for HCC as well. Here, the findings are promising, and the initial evidence confirmed that HMGB3 is one of the key molecules in the HMGB family. Future studies should clarify the molecular mechanisms of the upregulation of HMGB3 expression and its important role in hepatocarcinogenesis to elucidate that HMGB3 might promote proliferation of HCC cells and tumor growth by regulating cell cycle and DNA replication pathways.

**Article Highlights**

***Research background***

Hepatocellular carcinoma (HCC) still is one of the most common and rapidly fatal malignancies worldwide with a multi-factorial, multistep, complex process, and poor prognosis. Early diagnosis of HCC at early stage still is of the utmost importance. This study tried to find a new molecular biomarker to monitor the malignant transformation of hepatocytes.

***Research motivation***

Although serum alpha fetoprotein (AFP) level is a useful tumor marker for the detection and monitoring of HCC, the false-negative rate with AFP level alone may be as high as 40% for patients with early stage HCC. Even in patients with advanced HCC, the AFP levels may remain normal in 15~30% of the patients. New specific markers,such as circulating HS-GGT,HS-AFP or AFP-L3,miRNA,GPC-3, and GP73, have been developed to improve the sensitivity, specificity, early detection, and prediction of prognosis. However, the overall results have been unsatisfactory.

***Research objectives***

The most urgent needs are to find sensitive markers for early diagnosis or monitor postoperative recurrence, and to give adequate treatment for HCC. It has many characteristics, such as fast infiltrating growth, metastasis in early stage, high-grade malignancy, and poorly therapeutic efficacy, thus the prognosis is poor and early detection is of the utmost importance. The present study focused on exploring the relationship between dynamic expression of HMGB-3 and malignant transformation of hepatocytes.

***Research methods***

Dynamic models of rat hepatocarcinogenesis were made to investigate the characteristics from high mobility group box 1 (HMGB1) to HMGB4 expression. HMGB3 up-regulating expression presented with a trend confirmed at protein level by immunohistochemistry or Western blotting and at mRNA level by real time PCR. Human HMGB3 were evaluated using bioinformatics databases and its mechanisms were analyzed *in vitro* or xenograft growth *in vivo*.

***Research results***

Among the HMGB family, the expression characteristics from HMGB1 to HMGB4 were investigated during the dynamic alterations from normal rat hepatocytes to cancer formation. The abnormal HMGB3 expression was first observed and HMGB3 presented up-regulating expression with a trend during the malignant transformationof liver cells *in vivo*.

***Research conclusions***

The abnormality of liver HMGB3 expression was found by dynamic model of hepatocytes malignant transformation with the alterations of rat liver histopathology. Also, the new discovery has been confirmed by human HCC tissues of Bioinformatic databases and its regulating mechanism, suggesting that HMGB3 should be a novel insight for early diagnosis at stage or molecular therapy of HCC.

***Research perspectives***

HMGB3 has been confirmed as one of the key molecules in the HMGB family with HCC development. However, the molecular mechanisms of the upregulation of HMGB3 expression and its important role in hepatocarcinogenesis should be clarified in promoting proliferation of HCC cells and tumor growth and regulating cell cycle and DNA replication pathways in future.

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**Specialty type:** Gastroenterology and hepatology

**Country of origin:** China

**Peer-review report classification**

Grade A (Excellent): A

Grade B (Very good): 0

Grade C (Good): C

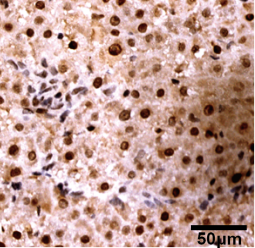
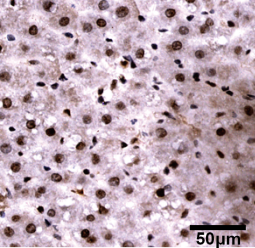
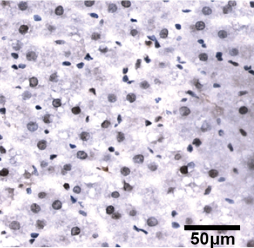
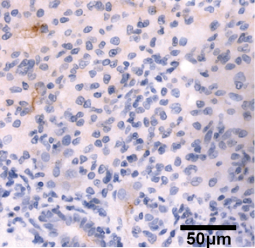
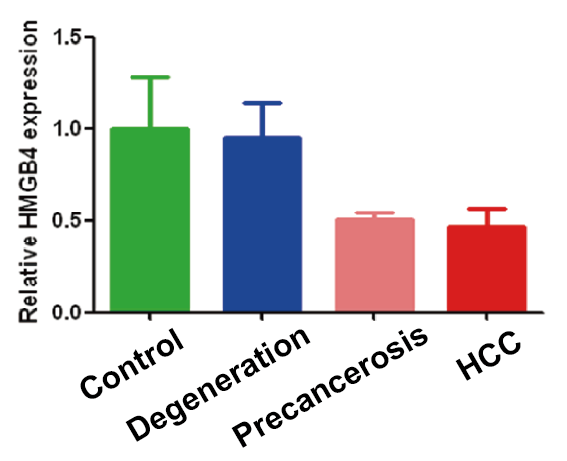
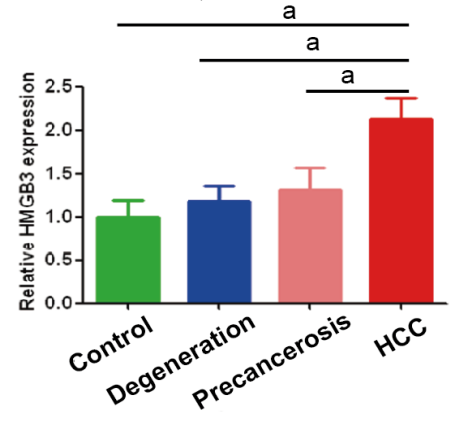
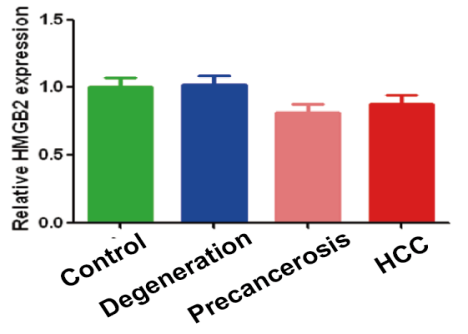
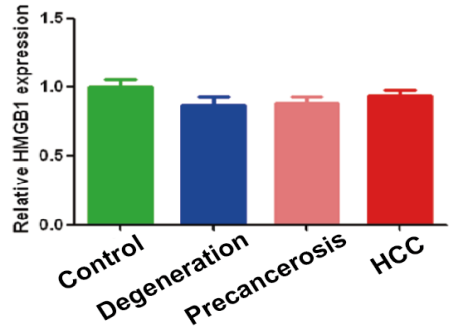
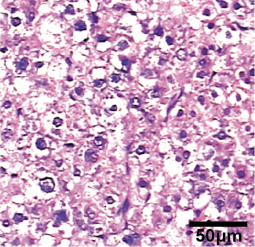
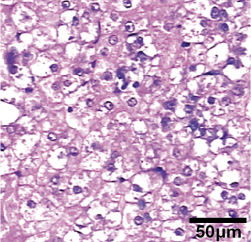
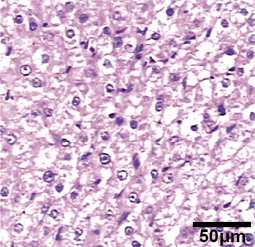
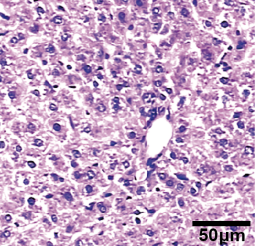
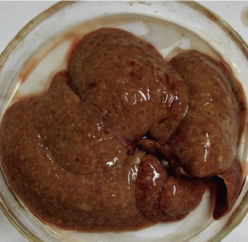
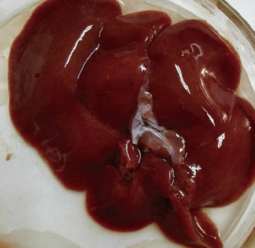
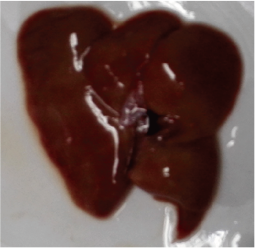
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**Table 1 Primers for real-time polymerase chain reaction**

|  |  |  |  |
| --- | --- | --- | --- |
| **Gene symbol** | **Species** | **Primer sequence (5’-3’)** | **Location** |
| *HMGB1* | Rat | F: GCTGACAAGGCTCGTTATGAA | 186-205 |
| R: CCTTTGATTTTGGGGCGGTA | 381-361 |
| *HMGB2* | Rat | F: CGGGGCAAAATGTCCTCGTA | 28-47 |
| R: ATGGTCTTCCATCTCTCGGAG | 155-135 |
| *HMGB3* | Rat | F: AGGTGACCCCAAGAAACCAAA | 9-29 |
| R: TCAGCAAAATTGACGGGAACC | 119-99 |
| *HMGB4* | Rat | F: AGACCAGCTAAGGCCCAAG | 12-30 |
| R: CCTTTTCGTGCTTTGAGATGGAT | 172-150 |
| *HMGB3* | Human | F: CCAAAGGGCAAGATGTCCG | 25-43 |
| R: TTGACAGGGACCTCTGGGTTT | 110-90 |
| *CCNB1* | Human | F: AATAAGGCGAAGATCAACATGGC | 43-65 |
| R: TTTGTTACCAATGTCCCCAAGAG | 153-131 |
| *FEN1* | Human | F: ATGACATCAAGAGCTACTTTGGC | 62-84 |
| R: GGCGAACAGCAATCAGGAACT | 142-122 |
| *PCNA* | Human | F: CCTGCTGGGATATTAGCTCCA | 77-97 |
| R: CAGCGGTAGGTGTCGAAGC | 185-167 |

HMGB: high mobility group box; FEN1: flap structure-specific endonuclease 1; PCNA: proliferating cell nuclear antigen; F: forward primer; R: reverse primer.



**Control Degeneration Precancerosis HCC**

**H&E Liver**

**Live**

**A**

**B**

**C**

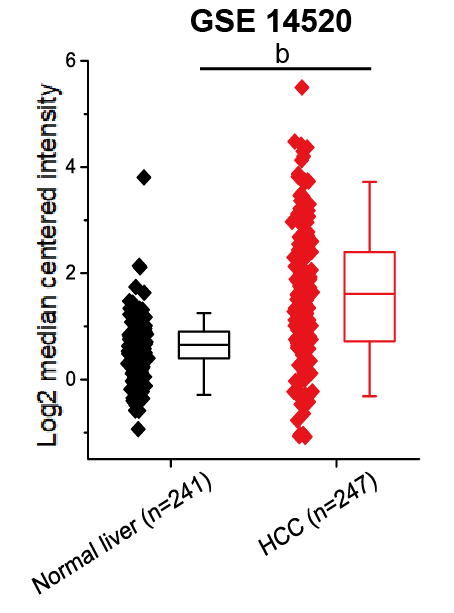
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**E**

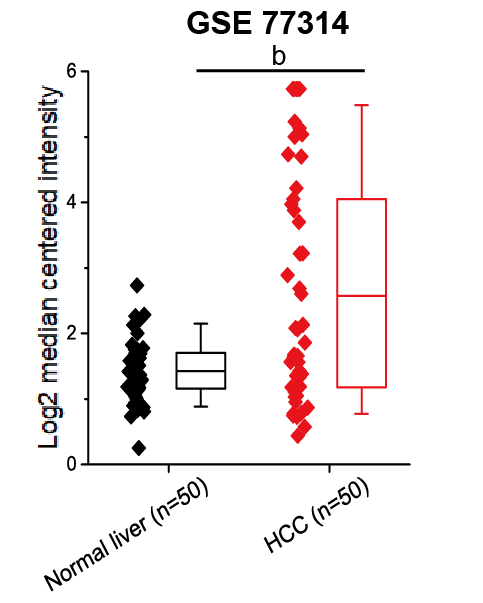
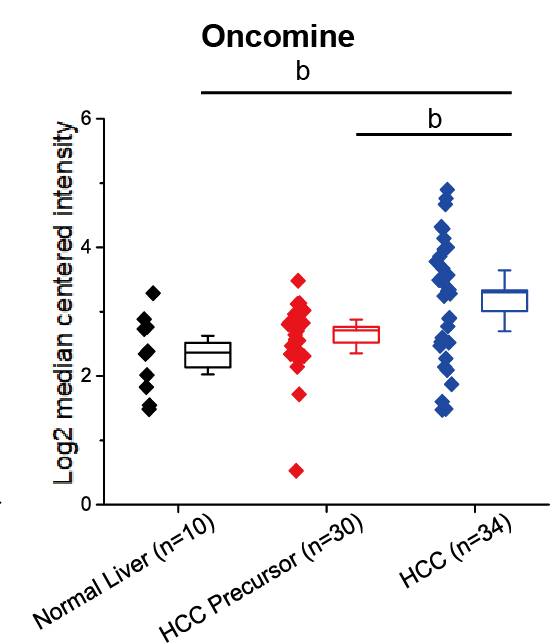
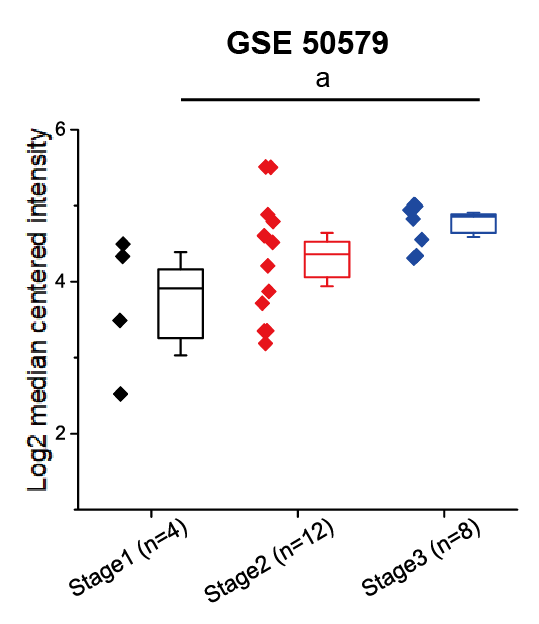
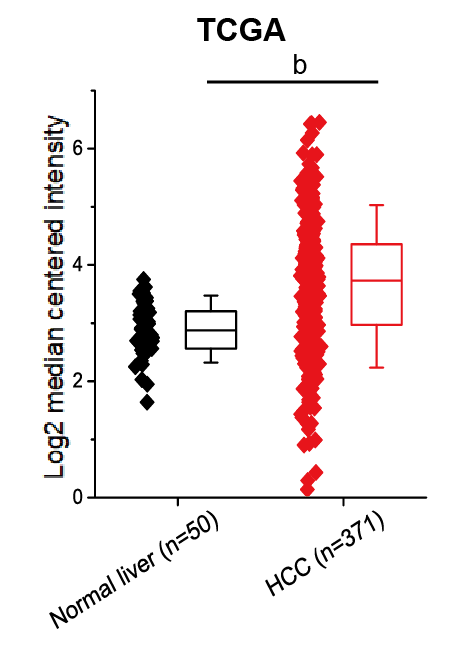
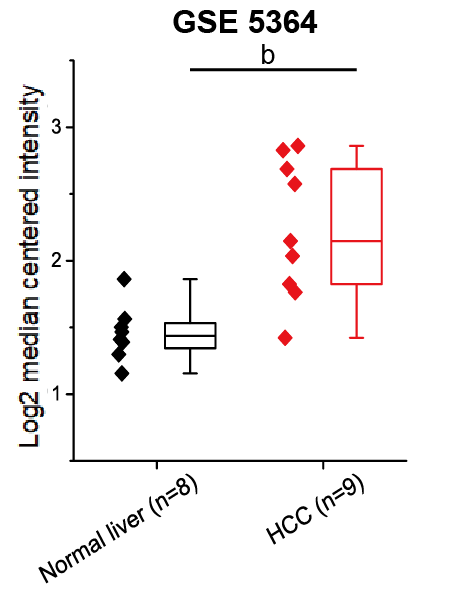
**F**

**Control Degeneration Precancerosis HCC**

**Figure 1 Dynamic up-regulating *HMGB3* in rat hepatocarcinogenesis.** Rat hepatocarcinogenesis models were successfully made by consistent 2-AAF intake. A: the dynamic alterations of liver morphology (up) and H&E staining (down) of liver tissues in rat hepatocarcinogenesis. The livers of rat model according to the results of rat liver H&E staining were divided into normal control, degeneration, precancerosis, and HCC group, respectively. B-E: the dynamic alterations of the HMGB family at mRNA level in models were detected by RT-qPCR. B: HMGB1 mRNA; C, HMGB2 mRNA; D, HMGB3 mRNA; E: HMGB4 mRNA; Each band was presented as a relative value normalized to normal controls (*n* = 6). F: the immunohistochemical staining of rat HMGB3 expression in different groups, respectively. a*P* < 0.05. 2-AAF: 2-acetylaminofluorene; H&E: hematoxylin and eosin; HMGB: high mobility group-box; RT-qPCR: reverse transcription-quantitative polymerase chain reaction.



**A**



**B**

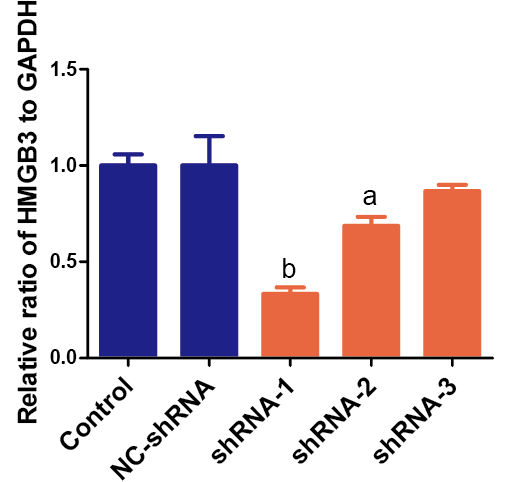
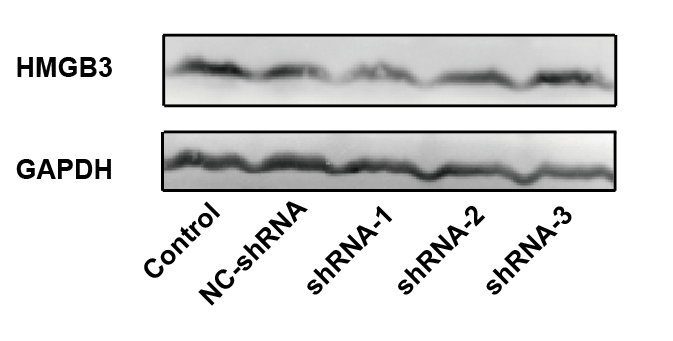
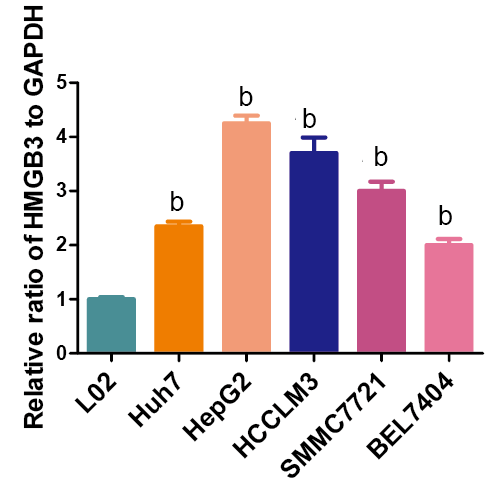
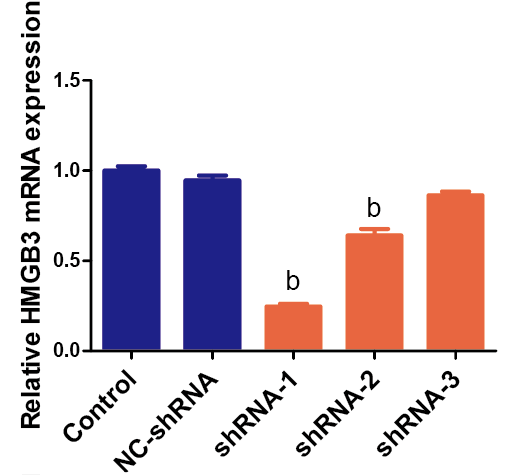
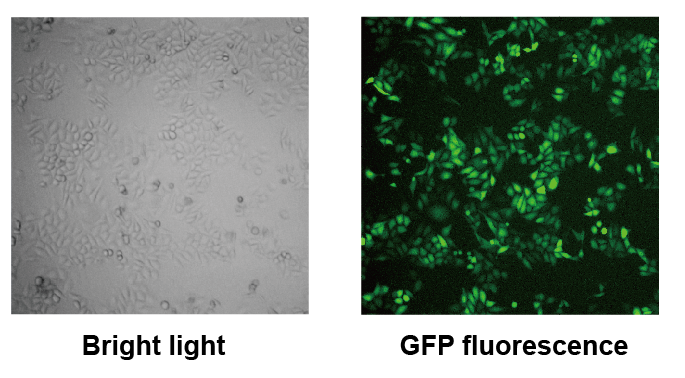
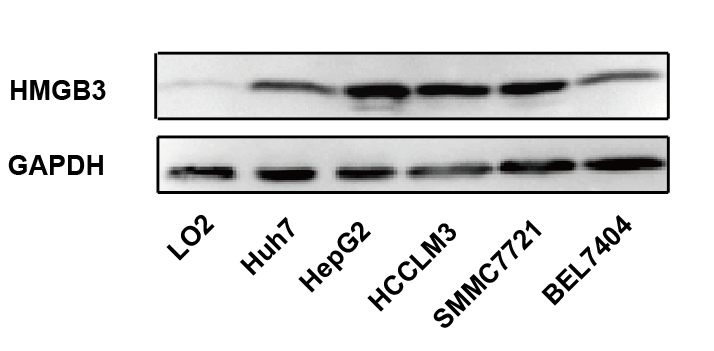
**C**

**D**

**E**

**F**

**Figure 2 *HMGB3* mRNA expression related to HCC by bioinformatic databases**. Comparative analysis of human normal livers (*n* = 359) and HCC (*n* = 765) tissues from bioinformatics databases suggested that the up-regulation of hepatic HMGB3 mRNA might be involved in HCC progression. The data of HMGB3 mRNA in HCC or normal liver were extracted from A: GSE-14520; B: GSE-5364; C: GSE-77314; and E: GSE-50579; D: the HMGB3 mRNA in TCGA database; F: the HMGB3 mRNA in Oncomine database. GSE, and GEO Series; TCGA, the Cancer Genome Atlas. Values were presented as Log2 median centered intensity. a*P* < 0.05; b*P* < 0.01.



**A**

**B**

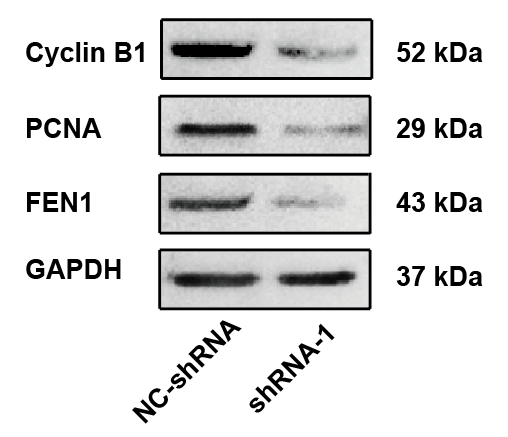
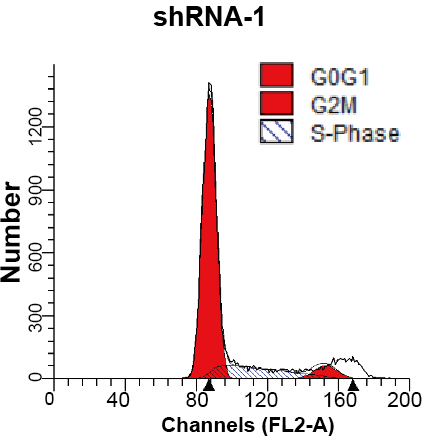
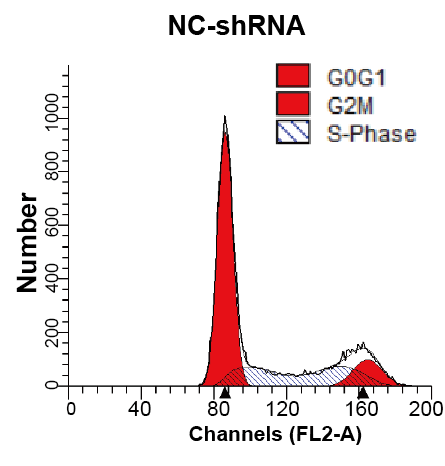
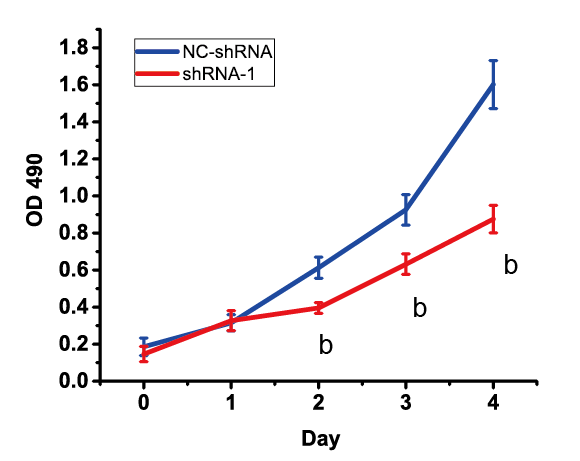
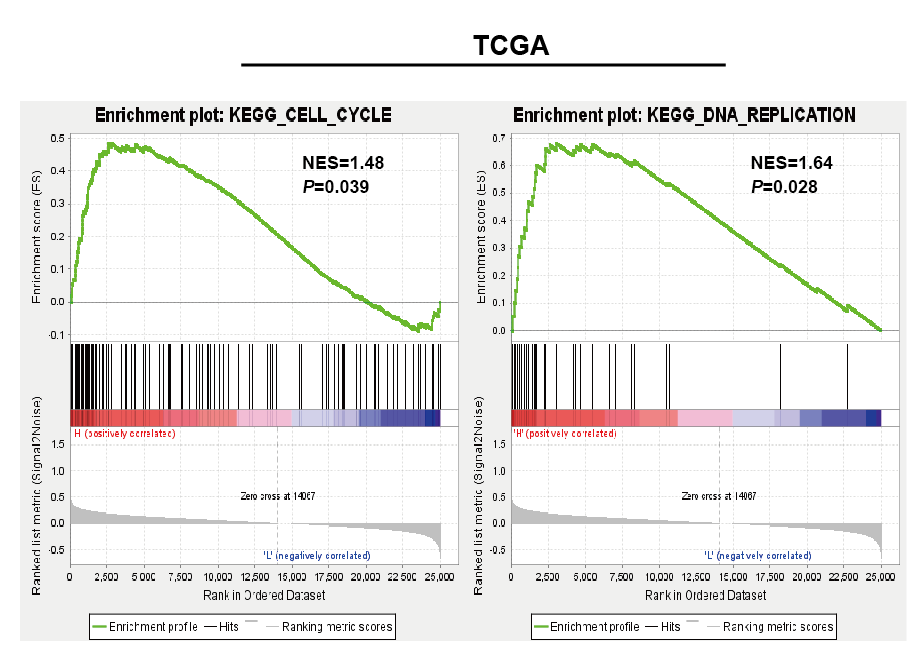
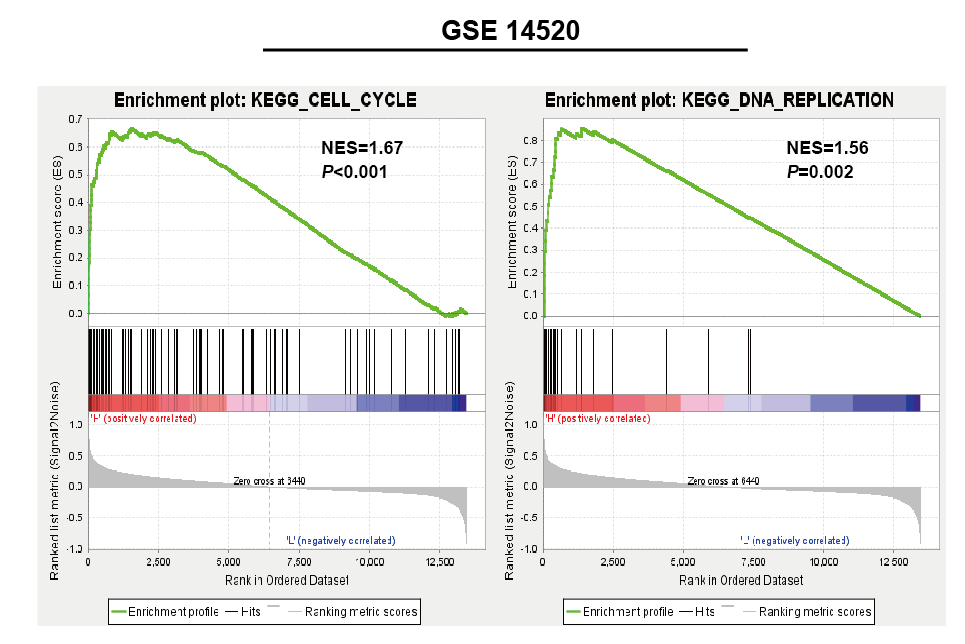
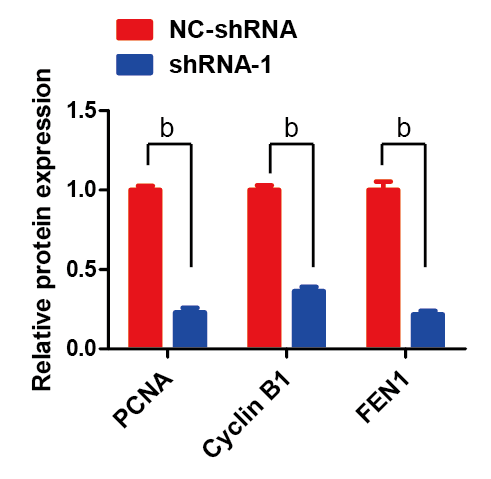
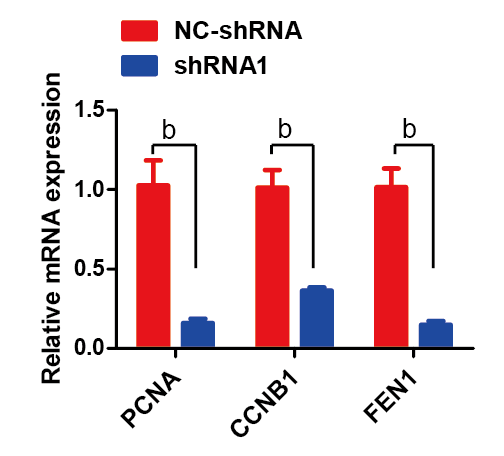
**C**

**D**

**E**

**F**

**Figure 3 Silencing *HMGB3* in hepatocellular carcinoma cell lines**. A: the protein expression of HMGB3 was detected in different HCC cell lines (Huh7, HepG2, HCCLM3, SMMC7721, and BEL7404), and normal hepatocytes L02 using western blotting. GAPDH was used as an internal reference. B: each bar represents the corresponding intensity in A normalized to GAPDH. C, representative morphology of HepG2 cells transfected with GFP-labeling shRNA in bright light and fluorescence. D: RT-qPCR was performed to detect HMGB3 mRNA levels in HepG2 cells transfected with different shRNAs and control. Relative value of HMGB3 was calculated according to 2−ΔΔCt method. E: western blotting was conducted to analyze the HMGB3 protein expression in cells of shRNA-transfected group and control group. F: each bar represents the corresponding intensity in E normalized to GAPDH. a*P* < 0.05; b*P* < 0.01.



**A**

**B**

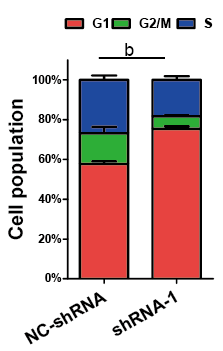
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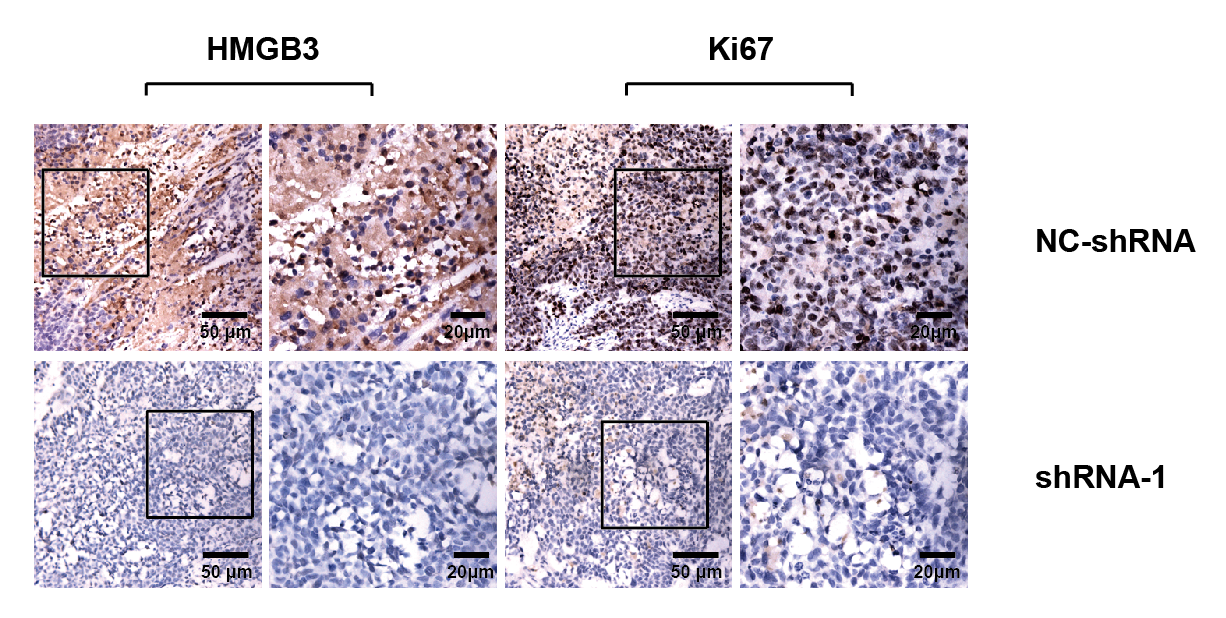
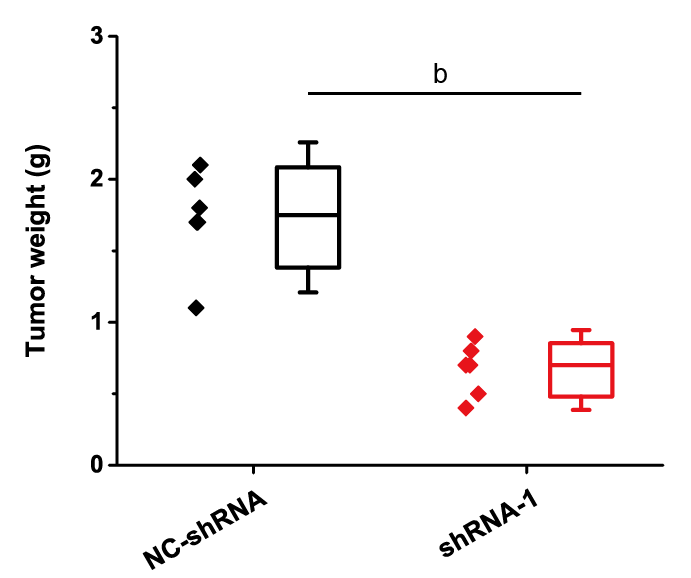
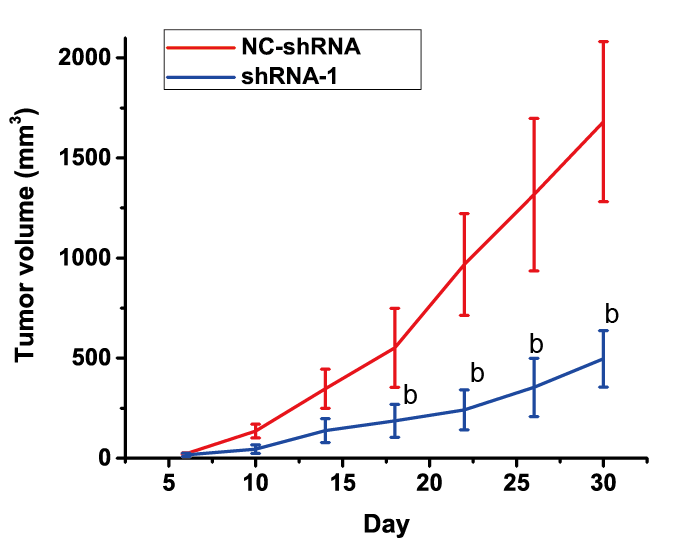
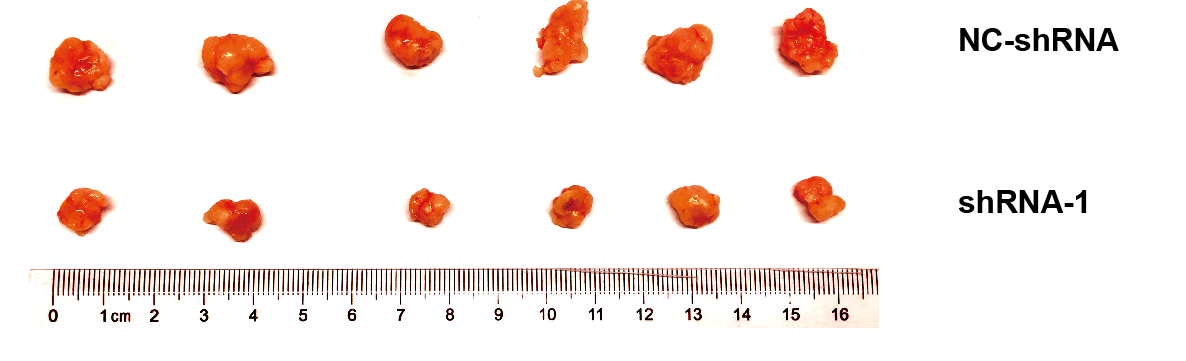
**E**

**F**

**G**



**Figure 4 Knockdown *HMGB3* inhibited cell cycle and proliferation of hepatocellular carcinoma cells.** A: gene set enrichment analysis (GSEA) was conducted to sort the pathways according to HMGB3 expression in GSE14520 and TCGA. Cell cycle and DNA replication pathways were found significantly correlated with HMGB3 expression. B: proliferation of HepG2 cells transfected with shRNA-1 and NC-shRNA was detected using MTT methods. C: cell cycle of HepG2 cells were analyzed after transfection with shRNA using flow cytometry. D: percentage columns represent the distribution of cell cycle in corresponding groups. E: according to the GSEA analysis, 3 genes (CCNB1, PCNA, and FEN1) involved in cell cycle and DNA replication were detected after shRNA transfection using RT-qPCR. F: western blotting was conducted to discover the protein expression of cyclin B1, PCNA, and FEN1 in NC-shRNA and shRNA-1 group. G, each bar represents the corresponding intensity in F normalized to GAPDH. b*P* < 0.01.



**A**

**B**

**C**

**D**

**Figure 5 Silencing *HMGB3* suppressed growth of xenograft tumor.** HepG2 cells transfected with NC-shRNA and shRNA-1 were subcutaneously injected into mice. A: the morphology of xenograft tumor in the NC-shRNA or shRNA-1 group at 30th day; B: the growth curves of tumors derived from HepG2 cells in the NC-shRNA or shRNA-1 group; C: the weight of xenograft tumor in the NC-shRNA or shRNA-1 group; and D: the immunochemical staining of HMGB3 and Ki67 in xenograft tumor tissues in the NC-shRNA or shRNA-1 group. b*P* < 0.01.