

Original Article

Correlation of HMGB1 expression to progression and poor prognosis of adenocarcinoma and squamous cell/adenosquamous carcinoma of gallbladder

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Abstract: HMGB1 (High mobility group box 1) expressions in adenocarcinoma (AC) and squamous cell/adenosquamous (SC/ASC) carcinoma of gallbladder, as well as its prognostic significance, have not yet been evaluated. We investigated HMGB1 expression in 80 cases of AC gallbladder cancer and 52 cases of SC/ASC gallbladder cancer. Survival information was concomitantly collected. The association of HMGB1 expression with clinicopathological characteristics and the possible prognostic role of HMGB1 for two aforementioned subtypes of gallbladder cancers were also analyzed. siRNA technique was utilized to explore the role of HMGB1 in proliferation and invasion of gallbladder cancer cells *in vitro*. HMGB1 overexpression is present in AC and SC/ASC gallbladder cancers. HMGB1 expression significantly associates with growth and metastasis of AC and SC/ASC gallbladder cancers. *In vitro* cell experiments based on siRNA demonstrated that HMGB1 downregulation inhibits proliferation and invasion of gallbladder cancer cells. Kaplan-Meier analysis revealed that HMGB1 expression is negatively associated with overall survival time of patients with AC or SC/ASC gallbladder cancer. Cox multivariate analysis confirmed that HMGB1 is an independent risk factor for survival of patients with AC or SC/ASC gallbladder cancer. HMGB1 overexpression closely correlates with progression and poor prognosis of AC and SC/ASC gallbladder cancers.

Keywords: Gallbladder cancer, squamous cell carcinoma, adenosquamous carcinoma, HMGB1, prognosis

Introduction

Gallbladder cancer is the most aggressive malignancy of biliary tract characterized by dismal prognosis and high mortality [1]. It is generally diagnosed at advance stage with serosal invasion and metastasis to other organs because absence of noticeable clinical signs or symptoms in early stage [2]. Most gallbladder cancer of early stage is accidentally discovered during or after cholecystectomy. In comparison to palliative chemotherapy and radiotherapy, only surgical resection serves as an effective strategy for improved survival of patient with advanced gallbladder cancer [3].

Histopathologically, over 90% of gallbladder cancer is adenocarcinoma (AC) [4], other subtypes, including mucinous, papillary and squa-

mous cell/adenosquamous (SC/ASC) subtypes, are rarely seen in clinical practice. SC/ASC subtype accounts for up to 10% of various gallbladder cancers [5]. However, only limited information is currently available regarding the clinicopathological and biological characteristics of SC/ASC gallbladder cancer since most prior reports are either single case reports or analyses of small sample size [5]. Studies of large sample are therefore warranted to promote a better understanding about SC/ASC gallbladder cancer. Although several biomarkers have been reported to associate with progression, invasion, metastasis and prognosis of AC gallbladder cancer [6-8], their clinical applications still remain unsatisfactory. More importantly, biomarker related to progression and prognosis of SC/ASC gallbladder cancer has not yet been fully explored.

Prognostic role of HMGB1 for gallbladder cancer

HMGB1, a highly-conserved yet versatile nuclear protein, is originally discovered as a chromatin-associated factor that binds DNA and facilitates gene transcription [9]. However, in addition to the nuclear function, HMGB1 is also found in the cytoplasm and stroma cells, and able to act as an extracellular signaling molecule in a variety of biological processes, including inflammation, cell migration and tumor metastasis [9]. Some researchers have previously reported that overexpression or cytoplasmic location of HMGB1 correlates with proliferation and metastasis of many malignancies [10-12]. High expression of HMGB1 is present in different solid tumors, such as gastric cancer [13], colon cancer [14], nasopharyngeal cancer [15] and breast cancer [16]. Aberrant expression of HMGB1 also predicts a poor prognosis in some cancer types [17-19]. However, HMGB1 expressions in tissues of AC and SC/ASC gallbladder cancers, as well as its prognostic significance, have not yet been evaluated before.

Considering the important role of HMGB1 in tumor progression, invasion and metastasis, we first investigated the expression of HMGB1 in 80 cases of AC gallbladder cancer and 52 cases of SC/ASC gallbladder cancer by immunohistochemistry. The association of HMGB1 expression with various clinicopathological characteristics and the possible prognostic role of HMGB1 for two aforementioned subtypes of gallbladder cancer were also analyzed. siRNA (Small interfering RNA) was additionally utilized to explore the role of HMGB1 in proliferation and invasion of gallbladder cancer cells *in vitro*.

Materials and methods

Case selection

This study was approved by The Ethic Committee for Human Research of Fujian Medical College, and conducted in accordance with the Helsinki Declaration. Written informed consents were obtained from all participants. A total of 132 cases of gallbladder cancer that underwent surgical resection or biopsy were recruited from First Hospital of Quanzhou during January 1997 and December 2013. All patients received no prior chemotherapy and radiotherapy before the surgeries. Of 132 cases of gallbladder cancer, 80 were diagnosed as AC and 52 were SC/ASC. Twenty cases of cholelithiasis that under-

went simple cholecystectomy were additionally included as the control. Immediately after surgical removal, tissue samples were fixed in 4% formaldehyde, dehydrated through graduated alcohol and then paraffin-embedded for subsequent immunohistochemistry. All diagnoses were based on histopathological examinations and clinical findings. Detailed clinicopathologic characteristics of all specimens were obtained from medical records and presented in [Table S1](#) and [Table S2](#) (See Supplementary Materials, [Table S1](#) and [Table S2](#)). Survival information was collected through letters and phone calls.

Chemicals and antibodies

Mouse anti-human HMGB1 monoclonal antibody was acquired from Santa Cruz Biotechnology Company (California, USA). Mouse anti-human β -actin, P53, Nm23, Ki-67, ER, PR and Her-2 polyclonal antibodies were obtained from Abcam (Massachusetts, USA). HRP-conjugated goat anti-mouse secondary antibody was procured from Zhongshan Biotechnology Company (Beijing, China).

Immunohistochemistry

Briefly, 4 μ m thick sections were cut, deparaffinated in xylene and rehydrated through graduated alcohol. The sections were heated for 20 min in citrate buffer (PH 6.0, Maixin, China) in microwave oven to retrieve antigens. Endogenous peroxidase activity was inactivated in 3% hydrogen peroxide (Maixin, China) for 10 min in darkness, and then blocked in Ultra V Block (Lab Vision Corporation, USA) for 30 min. The sections were incubated with primary antibody in a humid chamber at 4°C overnight. The primary antibody was removed on the following day and the slides were rinsed in PBS (PH 7.0, Maixin, China) and incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Immunohistochemical labeling was revealed using the DAB-Chromogen System (Dako Laboratories, USA) in accordance with the user manual. Substitution of primary antibody with PBS was served as blank control. Microphotographs were captured using an Olympus microscope (Olympus, Japan).

Immunohistochemical scoring

Immunostaining of HMGB1 was analyzed using a semi-quantitative scoring method in which

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the extent and intensity of immunostaining were concurrently evaluated [20]. In order to explore the association of HMGB1 expression with clinicopathological characteristics and survival, we defined moderate and strong staining as positive, the others as negative. For each section, a total of 800 cells in 20 random microscopic fields at the magnification of $\times 200$ were evaluated. The immunostaining of HMGB1 was evaluated by two independent pathologists in a blinded manner. A consensus score was achieved by reevaluation and discussion if there were any discrepancies.

Cell culture

Two human gallbladder cancer cell lines, TGBC1 and TGBC2, were obtained from Riken BRC Cell Bank (Ibaraki, Japan). Another human gallbladder cancer cell line GBC-SD was procured from Shanghai Institutes of Biological Sciences of China. Cells were maintained in Dulbecco's Modified Eagle's Medium (Gibco, USA) supplemented with 10% fetal bovine serum (Thermo Scientific, USA) and 1% penicillin-streptomycin cocktail (Sigma-Aldrich, USA). Cells were cultivated at 37°C in a humidified atmosphere of 5% CO₂.

Knockdown of HMGB1 expression by siRNA

siRNA against HMGB1 and the negative control siRNA were synthesized by and purchased from OriGene Technologies (OriGene, USA). siRNA transfection of GBC-SD cells was conducted using Lipofectamine 2000 kit (Invitrogen, USA) according to the user manual. 24 h after the transfection, cells were retrieved, resuspended and subjected to proliferation and invasion assays.

Assessment of cell proliferation

Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8, Dojindo, USA), in accordance with the manufacturer's instruction. The absorbance at 450 nm was detected with the Infinite M1000 microplate reader (TECAN, Austria).

Matrigel invasion assay

Cell invasion was examined by assessing the movement of cells into an artificial basal membrane of Matrigel-coated Transwell chambers

(BD Biosciences, USA) as described previously [21].

Immunoblot

Cells were harvested through trypsinization and centrifugation, lysed subsequently in RIPA buffer (Sigma-Aldrich, USA) supplemented with protease inhibitor cocktail (Roche, Germany). The lysate was then sonicated for 30 sec and centrifuged for 20 min. The supernatant was withdrawn and the protein concentration was assessed using BCA protein assay kit (Takara, Japan). Immunoblot was conducted as previously reported [22]. Immunoreactive signal was developed using the SuperSignal West Pico Chemiluminescence Kit (Thermo Scientific, USA), visualized and captured with the Chemi-Doc XRS Plus System (Bio-Rad, USA).

Statistical analysis

The data are expressed as mean \pm s.d., and analyzed by SPSS 16.0 (Illinois, USA). The χ^2 test or Fisher's exact probability test was used to investigate the statistical association between HMGB1 expression and clinicopathological characteristics. Kaplan-Meier method and time series test were applied to performed univariate survival analysis. Cox proportional hazards model was used to performed multivariate analysis and calculate the 95% confidence interval (95% CI). A *P* value of less than 0.05 was regarded as statistically significant.

Results

Comparison of HMGB1 expression in tissues of cholelithiasis gallbladder, AC gallbladder cancer and SC/ASC gallbladder cancer

With immunohistochemistry, we evaluated the expression of HMGB1 in tissues of AC and SC/ASC gallbladder cancers. Benign tissues of gallbladder with cholelithiasis were included as the control. As illustrated in **Figure 1B** and **Figure 1D**, immunoreactivity of HMGB1, a well-known nuclear protein, is predominantly localized to the nuclei but is also diffusively present in the cytoplasm in both AC and SC/ASC gallbladder malignancies. However, no cytoplasmic immunoreactivity of HMGB1 is observed in benign gallbladder tissues of cholelithiasis (**Figure 1F**). Moreover, as summarized in **Table S1** and **Table S2**, of 52 cases of SC/ASC gallbladder cancer,

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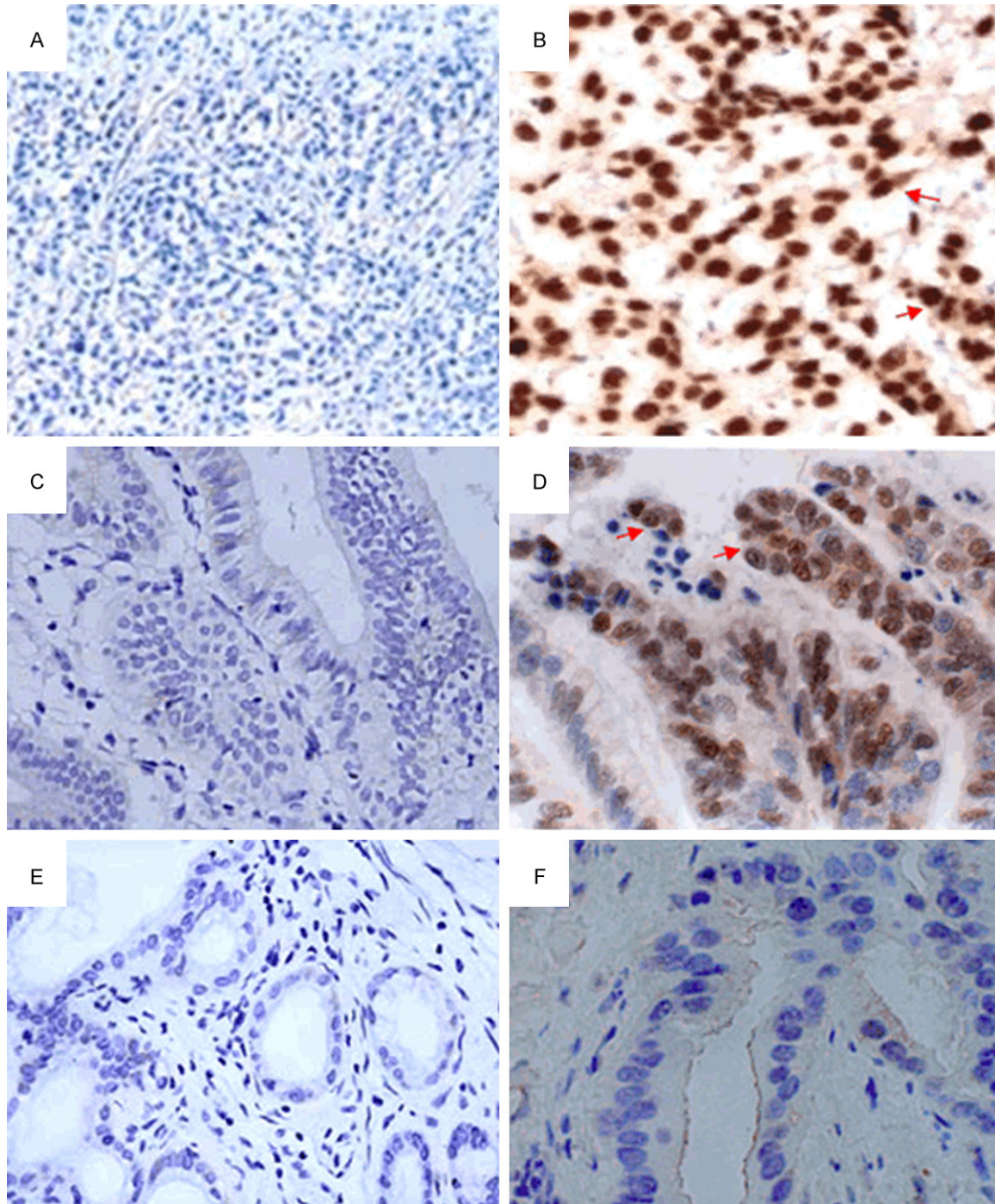


Figure 1. HMGB1 expression in tissues of cholelithiasis gallbladder, AC gallbladder cancer and SC/ASC gallbladder cancer. A. Blank control of Panel B, primary antibody was replaced by PBS. B. Positive HMGB1 expression in poorly-differentiated SC/ASC gallbladder cancer. HMGB1 staining is predominantly localized to the nuclei but also diffusely present in the cytoplasm (Red arrow). C. Blank control of Panel D. D. Positive HMGB1 expression in moderately-differentiated AC gallbladder cancer, HMGB1 staining is mainly found in the nuclei but also present in the cytoplasm (Red arrow). E. Blank control of Panel F. F. Negative HMGB1 expression in benign tissue of cholelithiasis gallbladder. PBS, Phosphate-buffered saline; AC, Adenocarcinoma; SC/ASC, Squamous cell/adenosquamous. Original magnification $\times 100$ for Panel A, C and E; Original magnification $\times 200$ for Panel B, D and F.

59.6% (31/52) were determined as positive for HMGB1 expression, showing a slight increase

as compared to that (48.8%, 39/80) of AC gallbladder cancer ($P>0.05$). In contrast, 0% (0/20)

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of cholelithiasis gallbladder are positive for HMGB1, displaying a markedly decrease when compared to the AC or SC/ASC counterpart (All $P<0.05$).

Association of HMGB1 expression with clinicopathological characteristics of AC gallbladder cancer

As shown in [Table S1](#), we found that significantly more cases of AC gallbladder cancer with maximal tumor diameter exceeding 3 cm are positive for HMGB1 expression as compared to those with maximal tumor diameter less than 3 cm (64.5% vs. 38.8%, $P<0.05$). In addition, HMGB1 expression also positively associates with expression of Ki-67, a reliable indicator of cell proliferation (53.8% for HMGB1⁺ Ki-67⁺ vs. 26.7% for HMGB1⁺ Ki-67⁻, $P<0.05$). These two lines of findings seem to indicate a promoting effect of HMGB1 on the growth of AC gallbladder cancer. Moreover, the percentage of AC gallbladder cancer concurrently positive for HMGB1 and ER is higher than that of AC gallbladder cancer only positive for HMGB1 (55.7% for HMGB1⁺ ER⁺ vs. 26.3% for HMGB1⁺ ER⁻, $P<0.05$). However, HMGB1 expression correlates negatively with the expression of Nm23, a protein capable of repressing metastasis (31.3% for HMGB1⁺ Nm23⁺ vs. 53.1% for HMGB1⁺ Nm23⁻, $P<0.05$). This correlation suggests that HMGB1 may be implicated in the metastasis of AC gallbladder cancer. HMGB1 expression shows no significant association with other clinicopathological characteristics of AC gallbladder cancer.

Association of HMGB1 expression with clinicopathological characteristics of SC/ASC gallbladder cancer

As presented in [Table S2](#), we discovered a negative correlation between HMGB1 expression and differentiation grade of SC/ASC gallbladder cancer; significantly more cases of poorly-differentiated SC/ASC gallbladder cancer are positive for HMGB1 expression (86.7%, 13/15) when compared to the counterpart of moderately-differentiated (55.6%, 10/18) or well-differentiated (42.1%, 8/19) SC/ASC gallbladder cancer ($P<0.05$). Furthermore, HMGB1 expression associates positively with lymph node metastasis (71.0% for HMGB1⁺ and lymph node metastasis vs. 42.9% for HMGB1⁺ but no lymph node metastasis, $P<0.05$). Similar to

the abovementioned findings, we found that HMGB1 expression also correlates positively with maximal tumor diameter (80.0% for HMGB1⁺ and maximal tumor diameter >3 cm vs. 46.9% for HMGB1⁺ and maximal tumor diameter ≤ 3 cm), ER expression (67.5% for HMGB1⁺ ER⁺ vs. 33.3% for HMGB1⁺ ER⁻) and Ki-67 expression (67.4% for HMGB1⁺ Ki-67⁺ vs. 22.2% for HMGB1⁺ Ki-67⁻), but correlated negatively with Nm23 expression (66.7% for HMGB1⁺ Nm23⁺ vs. 30.0% for HMGB1⁺ Nm23⁻) (All $P<0.05$). These evidences suggest that HMGB1 expression may be involved in growth and metastasis of SC/ASC gallbladder cancer.

Prognostic value of HMGB1 expression in patients with AC gallbladder cancer

Survival information of 80 cases of AC gallbladder cancer was obtained via letters or phone calls. 24 cases survived more than 1 year and the remaining 56 cases survived less than 1 year, with an average survival time of 12.1 months. Patients that survived more than 1 year have a lower percentage of HMGB1-positive cases as compared to those survived less than 1 year (45.8% vs. 73.2%, $P=0.019$). As summarized in [Table S3](#) (See Supplementary Materials, [Table S3](#)), Kaplan-Meier survival analysis showed that HMGB1 expression, tumor diameter, TNM stage, lymph node metastasis, ER expression, Ki-67 expression and Nm23 expression are associated with average survival time of patients with AC gallbladder cancer (All $P<0.05$). Average survival time for HMGB1-positive patients of AC gallbladder cancer is significantly shorter than that of HMGB1-negative patients (9.5 months vs. 20.1 months, $P<0.05$, [Table S3](#); **Figure 2B**). Cox proportional hazard regression model was additionally used to obtain a more accurate estimate about prognosis. As revealed in [Table S4](#) (See Supplementary Materials, [Table S4](#)), the results confirmed that tumor diameter (HR=1.58), HMGB1 expression (HR=3.05), TNM stage (HR=1.77), lymph node metastasis (HR=3.31) and Ki-67 expression (HR=2.43) are negatively associated with post-operative survival (All $P<0.05$), but Nm23 expression is positively associated with post-operative survival (HR=2.86, $P<0.05$), demonstrating that high HMGB1 expression is an independent prognostic factor for patient with AC gallbladder cancer.

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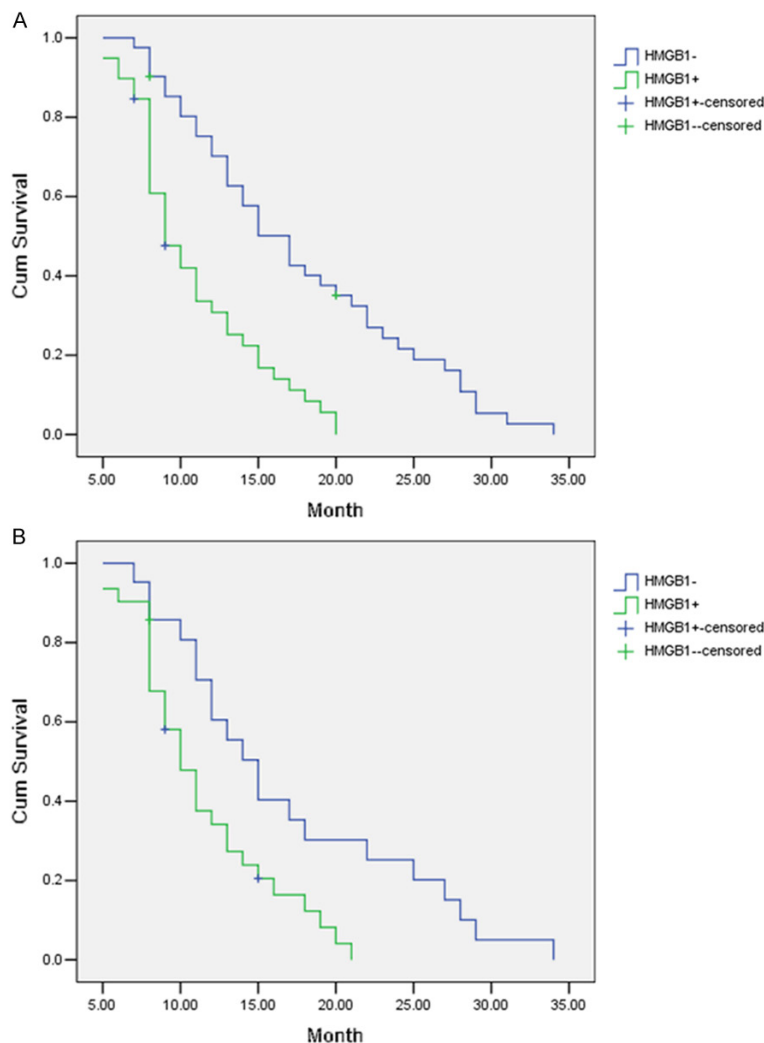


Figure 2. HMGB1 expression and survival in patients with SC/ASC or AC gallbladder cancer. A. Kaplan-Meier plot of overall survival in HMGB1-positive or HMGB1-negative patients with SC/ASC gallbladder cancer. B. Kaplan-Meier plot of overall survival in HMGB1-positive or HMGB1-negative patients with AC gallbladder cancer. AC, Adenocarcinoma; SC/ASC, Squamous cell/adenosquamous.

Prognostic value of HMGB1 expression in patients with SC/ASC gallbladder cancer

Survival information is available for 52 cases of SC/ASC gallbladder cancer. Of which, 16 cases survived longer than 1 year and the other 36 cases survived shorter than 1 year. The average survival time is 11.4 months. Patients that survived longer than 1 year have a lower percentage of HMGB1-positive cases when compared to those survived shorter than 1 year (50.0% vs. 81.3%, $P=0.001$). Kaplan-Meier survival analysis revealed that HMGB1 expression, differentiation grade, tumor diameter, TNM stage, lymph node metastasis, regional invasion,

ER expression, Ki-67 expression and Nm23 expression are significantly associated with average survival time of patients with SC/ASC gallbladder cancer (All $P<0.05$, [Table S5](#) [See Supplementary Materials, [Table S5](#)]). Average survival time for HMGB1-positive patients of SC/ASC gallbladder cancer is significantly shorter than that of HMGB1-negative patients (10.4 months vs. 21.5 months, $P<0.05$, [Table S5](#); **Figure 2A**). Cox proportional hazard regression model was also applied to gain a more precise estimate regarding the prognosis. The results confirmed that HMGB1 expression (HR=3.09), differentiation grade (HR=3.20), tumor diameter (HR=1.76), lymph node metastasis (HR=3.25) and Ki-67 expression (HR=2.30) are negatively associated with post-operative survival (All $P<0.05$, [Table S6](#) [See Supplementary Materials, [Table S6](#)]), whereas Nm23 expression is positively associated with post-operative survival (HR=3.81, $P<0.05$, [Table S6](#)), indicating that high HMGB1 expression is indeed an independent risk factor for prognosis of patient with SC/ASC gallbladder cancer.

In vitro effect of HMGB1 downregulation on proliferation and invasion of gallbladder cancer cells

In light of the abovementioned *in vivo* findings, we went on exploring the *in vitro* involvement of HMGB1 in gallbladder cancer. Three human gallbladder cancer cell lines, GBC-SD, TGBC-1 and TGBC-2, were selected. Immunoblot analysis showed that HMGB1 expression is indeed present in gallbladder cancer cells (**Figure 3A**). Next, HMGB1 expression was deliberately downregulated by siRNA technique (**Figure 3A**); influence of HMGB1 downregulation on proliferation and invasion was subsequently investigated by CCK-8 test and Matrigel invasion

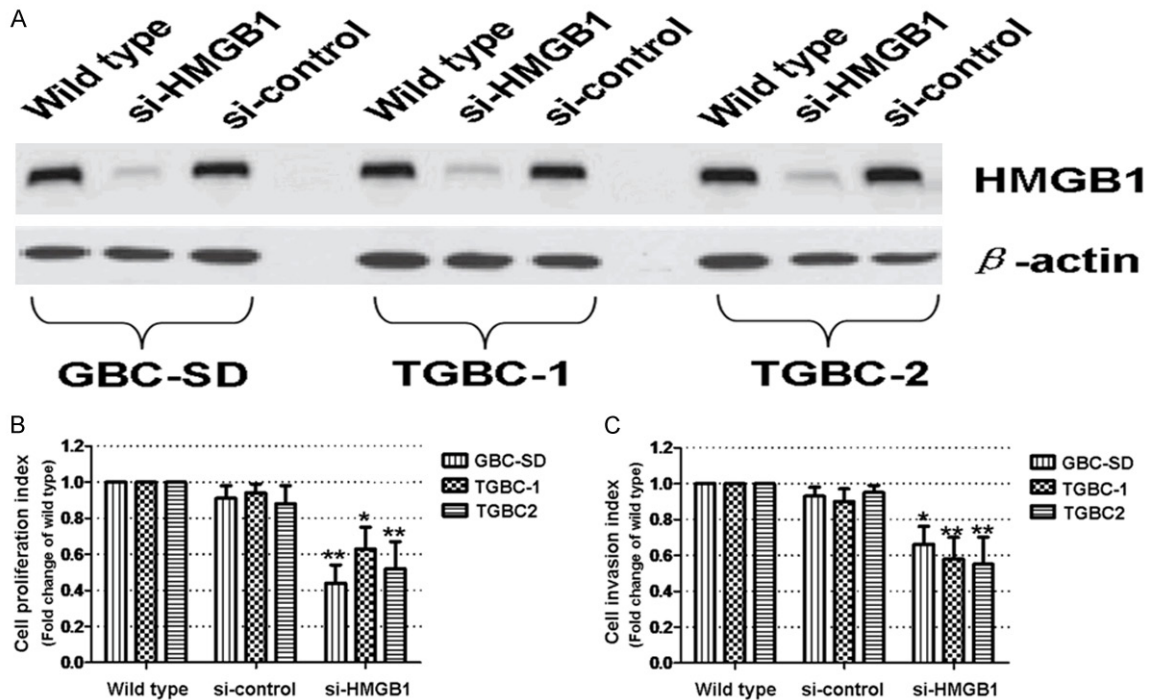


Figure 3. Effect of HMGB1 downregulation on proliferation and invasion of gallbladder cancer cells. A. siRNA-induced downregulation of HMGB1 in GBC-SD, TGBC-1 and TGBC-2 cells. β -actin was applied as the loading control. B. Effect of HMGB1 downregulation on proliferation of GBC-SD, TGBC-1 and TGBC-2 cells. Data are expressed as fold change of the wild type control (Which is assumed as 1). C. Effect of HMGB1 downregulation on invasion of GBC-SD, TGBC-1 and TGBC-2 cells. Data are similarly expressed as aforementioned. The results are representative of at least three separate experiments and shown as mean \pm s.d. * P <0.05; ** P <0.01, compared to the wild type control.

assay. We found that siRNA-induced downregulation of HMGB1 results in significant reduction in both proliferation and invasion of GBC-SD, TGBC-1 and TGBC-2 cells (Figure 3B and 3C, all P <0.05). Additionally, morphological result from Giemsa staining also confirmed the inhibitory effect on proliferation and invasion of GBC-SD, TGBC-1 and TGBC-2 cells (See Figure 4A-F for morphology of GBC-SD; Figures not shown for TGBC-1 and TGBC-2). Taken together, these *in vitro* evidences seem to suggest that elevated expression of HMGB1 may play a promoting role in progression and metastasis of gallbladder cancer.

Discussion

HMGB1 is a highly-conserved chromatin-binding chaperone that bends DNA and promotes access to transcriptional protein assemblies on specific DNA target [9]. HMGB1 has been reported to involve in various disease states, including sepsis [23], ischemia-reperfusion [24], arthritis [25], meningitis [26], neurode-

generation [27], senescence [28] and carcinogenesis [9]. Numerous previous studies demonstrated that HMGB1 expression is significantly elevated in a number of cancer types, including Non-Hodgkin lymphoma [29], colon cancer [18], prostate cancer [30] and cervical cancer [31].

Gallbladder cancer is a malignancy of high aggressiveness, rapid proliferation and poor prognosis. It is therefore necessary to explore reliable biomarkers associated with tumor formation, proliferation and survival. However, studies regarding clinicopathological and biological characteristics of gallbladder cancer, especially the rarely-seen SC/ASC type, are scarce owing to the low incidence. To date, only a few molecular markers have been reported to correlate with tumor invasion, aggression, metastasis and prognosis of gallbladder cancer [6-8]. Due to its rarity, studies of large sample size are thus warranted to systemically explore the clinicopathological and biological signatures of gallbladder cancer.

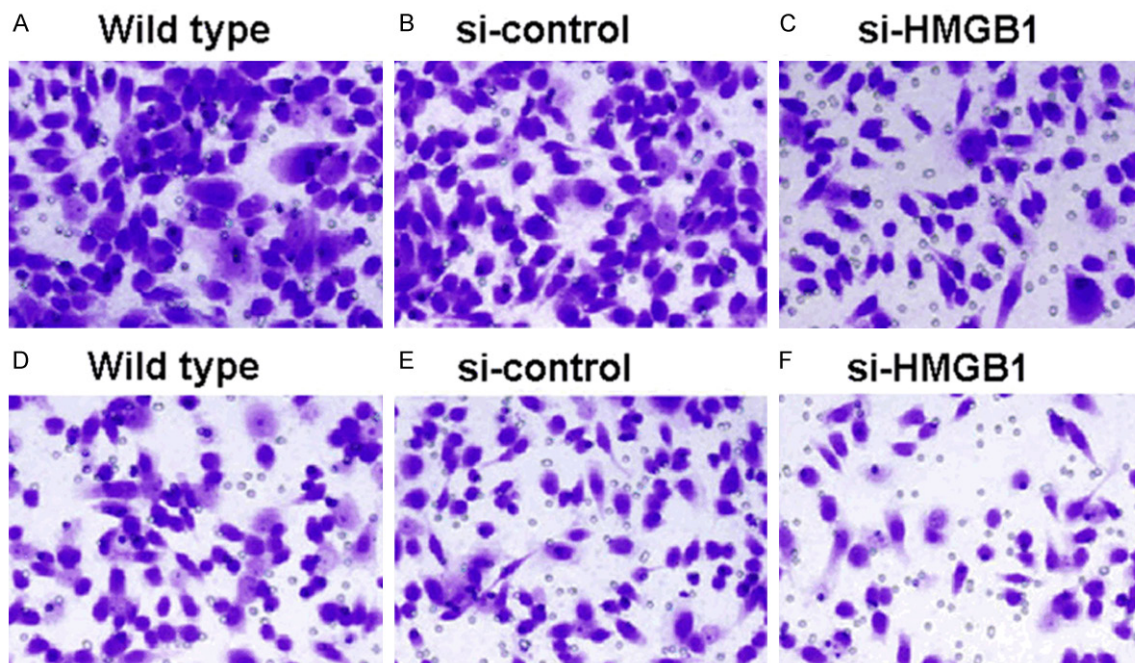


Figure 4. Proliferation and invasion of GBC-SD cells receiving different treatments. A-C. Proliferation of GBC-SD cells receiving indicated treatments. D-F. Invasion of GBC-SD cells receiving indicated treatments. Cells were stained with Giemsa solution, original magnification at $\times 200$.

In this study we recruited substantial specimens of both AC and SC/ASC gallbladder cancers, thus enabling us to gain a more accurate understanding about the molecular signatures of AC and SC/ASC gallbladder cancers. Although prior studies showed that HMGB1 is highly expressed in a numbers of human malignancies [6-8], its expression in different subtypes of gallbladder cancer has not yet been reported before. With immunohistochemistry, we measured the expression of HMGB1 and found that 59.6% of SC/ASC gallbladder cancer and 48.8% of AC gallbladder are respectively positive for HMGB1 expression (Table S1 and Table S2). This finding indicates that HMGB1 overexpression associates with gallbladder cancer. Previous studies demonstrated that HMGB1 expression is significantly elevated in a number of cancer types [18, 29, 31]. Expression of HMGB1 mRNA in prostate cancer is markedly higher than that of the normal prostate tissue [30]. Increased amount of HMGB1 expression is also found in malignant melanoma cells [32]. In agreement with these previous studies, our finding highlights the important role of HMGB1 in gallbladder cancer.

Moreover, we discovered that HMGB1 overexpression associates with tumor growth and

metastasis of both AC and SC/ASC gallbladder cancers (Table S1 and Table S2), a discovery also substantiated by the results of Kaplan-Meier survival analysis and Cox proportional hazard regression analysis (Tables S3-S6). Additionally, *in vitro* cell experiments based on siRNA technique demonstrated that downregulation of HMGB1 inhibits proliferation and invasion of gallbladder cancer cells (Figure 3 and Figure 4), confirming that HMGB1 overexpression correlates with growth and metastasis of gallbladder cancer. Albeit many previous studies have already explored the potential role of HMGB1 in tumor growth and metastasis, it still remains as a controversial hotspot. HMGB1 has been showed to play paradoxical roles in cell survival and death through regulation of multiple signal pathways [33]. HMGB1 exhibits dual functions, and can either be pro-tumor or anti-tumor, depending on the biological context [33]. The *in vitro* study by Palumbo showed that HMGB1 enhances migration and proliferation of adult and embryonic mesoangioblasts, and disrupts the barrier effect of endothelial monolayers [11]. Additionally, Kuniyasu found that cell growth, migration and invasion are markedly suppressed in Colo320 and WiDr carcinoma cells treated with HMGB1 antisense

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S-oligodeoxynucleotide when compared to the sense-treated cells [34]. Furthermore, Taguchi demonstrated in a animal study that inhibition of RAGE (Receptors for advanced glycation end-products)-HMGB1 interaction represses tumor growth and metastasis via activation of MAPK and NF- κ B pathways, and concurrently up-regulates the expressions of MMP2 and MMP9, proteins that degrade extracellular matrix proteins and promote tumor invasion and metastasis [35]. Todorova J and Pasheva E also proved in their recent study that suppression of HMGB1-RAGE interaction represses tumor growth and metastasis of lung cancer [36]. Our finding, together with these previous studies, seems to argue and underscore that HMGB1 expression may enhance tumor growth and promotes metastasis of AC and SC/ASC gallbladder cancers.

No previous studies have investigated the prognostic role of HMGB1 for gallbladder cancer. We are the first to analyze its prognostic value for gallbladder cancer. Kaplan-Meier analysis revealed that HMGB1 expression is negatively associated with overall survival of patients with AC or SC/ASC gallbladder cancer. Cox multivariate analysis also confirmed that HMGB1 is an independent risk factor in predicting overall survival time of patients with AC or SC/ASC gallbladder cancer. Taken together, these results seem to suggest a potential role of HMGB1 in promoting progression and malignancy of gallbladder cancer, as well as the possible application of HMGB1 as a novel prognostic biomarker for gallbladder cancer. In fact, the systematic review by Tang proposed that HMGB1 plays pivotal roles in cancer [33]. HMGB1 overexpression correlates with each of the hallmarks of cancer, including unlimited replication, re-angiogenesis, evasion of apoptosis, growth signal autonomy, insensitivity to anti-growth signal, pro-inflammation and tissue invasion and metastasis [33]. Survival analysis of patients with colorectal cancer demonstrated a significant poorer prognosis for patients with co-expression of RAGE and HMGB1 as compared to patients without the co-expression [37]. Furthermore, Chung also showed that HMGB1 level increases as gastric cancer progresses, and serum HMGB1 level associates significantly with invasion, lymph node metastasis, tumor size and poor prognosis [38]. These evidences clearly indicate a promoting role of HMGB1 in

progression and metastasis of gallbladder cancer, a notion that coincides well with our finding that HMGB1 overexpression associates with poor prognosis of patients with AC or SC/ASC gallbladder cancer.

In summary, our findings suggest that HMGB1 overexpression is closely associated with progression and poor prognosis of AC and SC/ASC gallbladder cancers. HMGB1 may be a novel prognostic biomarker and therapeutic target for gallbladder cancer.

Disclosure of conflict of interest

None.

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Table S1. The association of HMGB1 expression with various clinicopathological characteristics of patients with AC gallbladder cancer

Clinicopathological characteristics	Total NO.	HMGB1		P value
		Positive No. (%)	χ^2 value	
Gender				
Male	33	15 (45.5)	0.244	0.621
Female	47	24 (51.1)		
Age (Year)				
≤45	18	8 (44.4)	0.172	0.678
>45	62	31 (50.0)		
Differentiation grade				
Well-differentiated	30	11 (36.7)	0.858	0.651
Moderately-differentiated	27	13 (48.1)		
Poorly-differentiated	23	15 (65.2)		
Maximal tumor diameter (cm)				
≤3	49	19 (38.8)	5.035	0.025
>3	31	20 (64.5)		
Cholecystolithiasis				
No	36	17 (47.2)	0.061	0.805
Yes	44	22 (50.0)		
TNM stage				
I+II	29	11 (37.9)	2.439	0.295
III	38	20 (52.6)		
IV	13	8 (61.5)		
Lymph node metastasis				
No	34	15 (44.1)	0.508	0.476
Yes	46	24 (52.2)		
Regional invasion				
No	35	16 (45.7)	0.230	0.632
Yes	45	23 (51.1)		
Surgical method				
Radical	31	13 (41.9)	2.782	0.256
Palliative	27	12 (44.4)		
Without resection	22	14 (63.6)		
Her-2 expression				
Positive	30	16 (53.3)	0.404	0.525
Negative	50	23 (46.0)		
ER expression				
Positive	61	34 (55.7)	5.020	0.025
Negative	19	5 (26.3)		
PR expression				
Positive	59	29 (49.2)	0.015	0.904
Negative	21	10 (47.6)		
P53 expression				
Positive	64	32 (50.0)	0.200	0.655
Negative	16	7 (43.8)		
Ki-67 expression				
Positive	65	35 (53.8)	3.604	0.058
Negative	15	4 (26.7)		
Nm23 expression				
Positive	16	4 (31.3)	4.515	0.034
Negative	64	35 (53.1)		

Prognostic role of HMGB1 for gallbladder cancer

Table S2. The association of HMGB1 expression with various clinicopathological characteristics of patients with SC/ASC gallbladder cancer

Clinicopathological characteristics	Total NO.	HMGB1		P value
		Positive No. (%)	χ^2 value	
Gender				
Male	20	11 (55.0)	0.288	0.592
Female	32	20 (62.5)		
Age (Year)				
≤45	11	6 (54.5)	0.146	0.702
>45	41	25 (63.4)		
Differentiation grade				
Well-differentiated	19	8 (42.1)	7.102	0.029
Moderately-differentiated	18	10 (55.6)		
Poorly-differentiated	15	13 (86.7)		
Maximal tumor diameter (cm)				
≤3	32	15 (46.9)	5.609	0.018
>3	20	16 (80.0)		
Cholecystolithiasis				
No	24	14 (58.3)	0.030	0.862
Yes	28	17 (60.7)		
TNM stage				
I+II	15	6 (40.0)	3.661	0.056
III	28	18 (64.3)		
IV	9	7 (77.8)		
Lymph node metastasis				
No	21	9 (42.9)	4.109	0.043
Yes	31	22 (71.0)		
Regional invasion				
No	22	11 (50.0)	1.464	0.226
Yes	30	20 (66.7)		
Surgical method				
Radical	19	8 (42.1)	5.025	0.081
Palliative	18	11 (61.1)		
Without resection	15	12 (80.0)		
Her-2 expression				
Positive	21	13 (61.9)	0.077	0.782
Negative	31	18 (58.1)		
ER expression				
Positive	40	27 (67.5)	4.390	0.036
Negative	12	4 (33.3)		
PR expression				
Positive	38	23 (60.5)	0.049	0.825
Negative	14	8 (57.1)		
P53 expression				
Positive	42	25 (59.2)	0.001	0.978
Negative	10	6 (60.0)		
Ki-67 expression				
Positive	43	29 (67.4)	6.199	0.013
Negative	9	2 (22.2)		
Nm23 expression				
Positive	10	3 (30.0)	4.424	0.035
Negative	42	28 (66.7)		

Prognostic role of HMGB1 for gallbladder cancer

Table S3. Univariate analysis of survival for 80 patients with AC gallbladder cancer

Characteristics	Category	No.	Average survival time (Month)	95% CI (Month)	P value
HMGB1 expression	Positive	39	9.5	6.3-12.7	<0.05
	Negative	41	20.1	13.9-26.3	
Maximal tumor diameter	≤3 cm	49	25.6	15.3-35.9	<0.05
	>3 cm	31	12.0	8.5-15.5	
TNM stage	I+II	29	30.6	23.2-38.0	<0.05
	III	38	11.1	7.3-14.9	
	IV	13	7.6	5.4-9.80	
Lymph node metastasis	No	34	18.1	12.6-23.6	<0.05
	Yes	46	9.7	5.5-13.9	
ER expression	Positive	61	17.5	12.0-23.0	<0.05
	Negative	19	10.5	6.2-14.8	
Ki-67 expression	Positive	65	10.3	6.1-14.5	<0.05
	Negative	15	17.9	12.1-23.7	
Nm23 expression	Positive	16	18.1	12.6-23.6	<0.05
	Negative	64	9.7	5.6-13.8	

Table S4. Multivariate Cox regression analysis of survival for 80 patients with AC gallbladder cancer

Characteristics	Category	Hazard ratio (HR)	95% CI	P value
HMGB1 expression	Positive	3.05	1.10-8.47	<0.05
	Negative			
Maximal tumor diameter	≤3 cm	1.58	0.84-3.20	<0.05
	>3 cm			
TNM stage	I+II	1.77	1.01-3.03	<0.05
	III			
	IV			
Lymph node metastasis	No	3.31	1.41-7.73	<0.05
	Yes			
Ki-67 expression	Positive	2.43	1.25-4.73	<0.05
	Negative			
Nm23 expression	Positive	2.86	1.14-6.36	<0.05
	Negative			

Prognostic role of HMGB1 for gallbladder cancer

Table S5. Univariate analysis of survival for 52 patients with SC/ASC gallbladder cancer

Characteristics	Category	No.	Average survival time (Month)	95% CI (Month)	P value
HMGB1 expression	Positive	31	10.4	7.2-13.6	<0.05
	Negative	21	21.5	14.4-28.6	
Maximal tumor diameter	≤3 cm	32	24.4	14.1-34.7	<0.05
	>3 cm	20	13.2	8.9-17.5	
Differentiation degrade	Well-differentiated	19	29.5	22.7-36.3	<0.05
	Moderately-differentiated	18	11.4	7.9-14.9	
	Poorly-differentiated	15	6.7	4.3-9.1	
TNM stage	I+II	15	28.3	20.1-36.5	<0.05
	III	28	10.3	6.7-13.9	
	IV	9	7.4	5.1-9.7	
Lymph node metastasis	No	21	17.0	11.4-22.6	<0.05
	Yes	31	9.1	5.0-13.2	
Regional invasion	No	22	18.3	12.7-23.9	<0.05
	Yes	30	8.4	4.3-12.5	
ER expression	Positive	40	17.3	12.0-22.6	<0.05
	Negative	12	12.3	8.4-16.2	
Ki-67 expression	Positive	43	9.1	5.2-13.0	<0.05
	Negative	9	16.2	11.4-21.0	
Nm23 expression	Positive	10	16.3	10.3-22.3	<0.05
	Negative	42	8.5	4.3-12.7	

Table S6. Multivariate Cox regression analysis of survival for 52 patients with SC/ASC gallbladder cancer

Characteristics	Category	Hazard ratio (HR)	95% CI	P value
HMGB1 expression	Positive	3.09	1.19-8.06	<0.05
	Negative			
Differentiation degrade	Well-differentiated	3.20	1.08-9.83	<0.05
	Moderately-differentiated			
	Poorly-differentiated			
Maximal tumor diameter	≤3 cm	1.76	0.75-4.11	<0.05
	>3 cm			
TNM stage	I+II	1.65	0.65-1.65	<0.05
	III			
	IV			
Lymph node metastasis	No	3.25	1.37-7.72	<0.05
	Yes			
Ki-67 expression	Positive	2.30	1.12-4.74	<0.05
	Negative			
Nm23 expression	Positive	3.81	1.45-9.96	<0.05
	Negative			