Abstract. High mobility group box 1 (HMGB1) as a novel inflammatory molecule has been shown to be involved in a variety of cell physiological and pathological behaviors including immune response, inflammation and cancer. Evidence suggests that HMGB1 plays a critical role in the development and progression of multiple malignancies. However, the underlying molecular mechanisms for the HMGB1-mediated growth and invasion of gastric cancer have not yet been elucidated. The present study investigated the expression of HMGB1 in gastric adenocarcinoma (GAC) and the mechanisms by which it contributes to tumor growth and invasion. The correlation between HMGB1 expression and clinicopathological characteristics of GAC patients was assessed by immunohistochemical assay through tissue microarray procedures. The RNA and protein expressions of HMGB1 and downstream factors were detected by quantitative PCR and western blot assays; cell proliferation and invasion were determined by MTT, wound-healing and 3D-Matrigel assays, subcutaneous SGC-7901 tumor models were established to verify tumor growth in vivo. We demonstrated that, the expression of HMGB1 was significantly increased in the nucleus of GAC tissues compared with that in adjacent non-cancer tissues (88.6 vs.70.5%, P<0.001), and correlated with the metastatic lymph node of GAC (P=0.018). Furthermore, knockdown of HMGB1 by shRNA inhibited cell proliferative activities and invasive potential, and downregulated the expression of NF-κB p65, PCNA and MMP-9 in GAC cells (SGC-7901 and AGS). The tumor volumes in SGC7901 subcutaneous nude mouse models treated with Lv-shHMGB1 was significantly smaller than those of the nonsense sequence group. Taken together, these findings suggest that increased expression of HMGB1 is associated with tumor metastasis of GAC, and knockdown of HMGB1 suppresses growth and invasion of GAC cells through the NF-κB pathway in vitro and in vivo, suggesting that HMGB1 may serve as a potential therapeutic target for GAC.

Introduction

Gastric cancer is one of the most common malignancies worldwide, with an estimated 21,600 new cases and 10,990 deaths reported in United States in 2013 (1). Gastric cancer is also a genetic disease developing from a multi-step process. Single or multiple mutations in genes related to growth control, invasion and metastasis form the molecular genetic basis of malignant transformation and tumor progression (2). Thus, identification of key genes or targets related to tumorigenesis is crucial for the diagnosis and prevention of gastric cancer.

HMGB1 is a chromosome-binding protein that also acts as a damage-associated molecular pattern molecule. It has potent proinflammatory effects and is one of key mediators of organ injury (3) and the target of inflammation controlling (4). HMGB1 is involved in certain physiologic and pathologic conditions including cancer and is identified to be differentially expressed in prostate and ovarian cancers (5,6). The expression of HMGB1 has been reported in human malignant tumors of various differentiation levels (7), and it is upregulated in human lymphomas (8) and squamous cell carcinoma of head and neck (SCCHN) (9). Moreover, overexpression of HMGB1 correlates with tumor progression and poor prognosis in colorectal carcinoma (CRC) (10), and contributes to the malignant progression of SCCHN (11). HMGB1 enhances tumor migration by increasing α5β1 integrin expression through the RAGE/PI3K/Akt pathway (12), induces the overexpression of miR-221/-222, promotes growth of thyroid...
cancer cells (13), and activates TLR4 and RAGE signaling pathways inducing caspase-1 with subsequent production of many inflammatory mediators which in turn promotes cancer invasion and metastasis (14), suggesting that HMGB1 may be utilized as a diagnostic and therapeutic target for cancer (6,15). In addition, HMGB1 is released from tumor cells after chemotherapy-induced cytotoxicity, and induces autophagy promoting chemoresistance in leukemia cells (16), which is probably connected with activation of the PI3K/Akt/mTORC1 pathway (17).

Only few studies indicate that HMGB1 expression is down-regulated in the lung, lymph node and spleen tumor samples compared to their non-neoplastic counterparts (18), and the inversely associated with the infiltration of CD45RO+ T cells and prognosis in patients with stage IIIB colon cancer (19). HMGB1 functions as a tumor suppressor and radiosensitizer in breast cancer (20). To further elucidate the expression and function of HMGB1 in cancer, we examined the expression of HMGB1 in primary cancer tissues and adjacent non-cancer tissues derived from patients with primary GAC, and further investigated the effects of HMGB1 knockdown by shRNA on cell proliferation and invasion in vitro and in vivo. We hypothesized that HMGB1 expression was increased in GAC tissues and knockdown of HMGB1 suppressed growth and invasion of gastric adenocarcinoma (GAC) cells, suggesting and HMGB1 may serve as a potential therapeutic target for GAC.

Materials and methods

Materials. The GAC cell lines (SGC-7901 and AGS) used for experiments was obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China). Lentivirus-mediated HMGB1 shRNA vector, negative control vector, and virion-packaging elements were purchased from Genechem (Shanghai, China). Human GAC tissues and the corresponding adjacent non-cancer tissues (ANCT) were collected from Department of Gastrointestinal Surgery of Shanghai Six People's Hospital. The tissue microarray of gastric cancer was made by Shanghai Outdo Biotech Co. Ltd. (Shanghai, China). The antibody of HMGB1 was purchased from Cell Signaling Technologies (Boston, MA, USA). HMGB1 primer was synthesized by ABI (Framingham, MA, USA).

Drugs and reagents. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA); TRIzol Reagent and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA, USA); M-MLV Reverse Transcriptase was purchased from Promega (Madison, WI, USA); SYBR Green Master Mix was obtained from Takara (Otsu, Japan); and the ECL Plus kit was obtained from GE Healthcare (Piscataway, NJ, USA).

Clinical samples and data. Tissue microarray was prepared for IHC test. Human gastric cancer tissues and the corresponding ANCT were obtained from a biopsy in a total of 88 consecutive GAC cases admitted to our hospital from January 2007 to December 2012. The baseline characteristics of the patients before neo-adjuvant chemotherapy were recorded. The study was approved by Medical Ethics Committee of Shanghai Jiao Tong University and written informed consent was obtained from the patients or their parents before sample collection. Two pathologists independently reviewed all of the cases.

Tissue microarray. The advanced tissue arrayer (ATA-100, Chemicon International, Tamecula, CA, USA) was used to create holes in a recipient paraffin block and to acquire cylindrical core tissue biopsies with a diameter of 1 mm from the specific areas of the ‘donor’ block. The tissue core biopsies were transferred to the recipient paraffin block at defined array positions. The tissue microarrays contained tissue samples from 88 formalin-fixed paraffin-embedded cancer specimens with known diagnosis, and corresponding ANCT from these patients. The block was incubated in an oven at 45°C for 20 min to allow complete embedding of the grafted tissue cylinders in the paraffin of the recipient block, and then stored at 4°C until microtome sectioning.

Immunohistochemical staining. Anti-HMGB1 antibody was used for IHC detection of the expression of HMGB1 protein in tissue microarray. Tissue microarray sections were processed for IHC analysis of KiSS1 protein as follows. Immunohistochemical examinations were carried out on 3-mm thick sections. For anti-HMGB1 immunohistochemistry, unmasking was performed with 10 mM sodium citrate buffer, pH 6.0, at 90°C for 30 min. For anti-HMGB1 immunohistochemistry, antigen unmasking was not necessary. Sections were incubated in 0.03% hydrogen peroxide for 10 min at room temperature, to remove endogenous peroxidase activity, and then in blocking serum (0.04% bovine serum albumin, A2153, Sigma-Aldrich, Shanghai, China and 0.5% normal goat serum X0907, DAKO Corp., Carpinteria, CA, USA, in PBS) for 30 min at room temperature. Anti-HMGB1 antibody was used at a dilution of 1:200. The antibody was incubated overnight at 4°C. Sections were then washed three times for 5 min in PBS. Non-specific staining was blocked with 0.5% casein and 5% normal serum for 30 min at room temperature. Finally, staining was developed using diaminobenzidine substrate, and sections were counterstained with hematoxylin. Normal serum or PBS was used to replace anti-KiSS1 antibody in negative controls.

Quantification of protein expression. The expression of HMGB1 was semiquantitatively estimated as the total immunostaining scores, which were calculated as the product of a proportion score and an intensity score. The proportion and intensity of the staining was evaluated independently by two observers. The proportion score reflected the fraction of positive staining cells (0, none; 1, ≤10%; 2, 10% to ≥25%; 3, >25-50%; 4, >50%), and the intensity score represented the staining intensity (0, no staining; 1, weak; 2, intermediate; 3, strong). Finally, a total expression score was given ranging from 0 to 12. Based on the pre-analysis, HMGB1 expression was categorized into two groups: low-level HMGB1 expression (score 0-3) and high-level HMGB1 expression (score 4-12). The scoring was independently assessed by two pathologists.

Cell culture and transfection. GAC cells (SGC-7901 and AGS) were cultured in DMEM medium supplemented with 10% heat-inactivated FBS, 100 U/ml of penicillin, and 100 µg/
ml of streptomycin. Cells in this medium were placed in a humidified atmosphere containing 5% CO₂ at 37°C. Cells were subcultured at a 1:5 dilution in medium containing 300 µg/ml G418 (an aminoglycoside antibody, commonly used stable transfection reagent in molecular genetic testing). On the day of transduction, GAC cells were replated at 5x10⁴ cells/well in 24-well plates containing serum-free growth medium with polybrene (5 mg/ml). At 50% confluence, cells were transfected with recombinant experimental virus or control virus at the optimal MOI (multiplicity of infection) of 50, and cultured at 37°C and 5% CO₂ for 4 h. Then supernatant was discarded and serum containing growth medium was added. At 4 days of post-transduction, transduction efficiency was measured by the frequency of green fluorescent protein (GFP)-positive cells. Positive and stable transfectants were selected and expanded for further study. The HMGB1 shRNA virus vector-infected clone, the negative control vector-infected cells, and GAC cells (SGC-7901 and AGS) were named as Lv-shHMGB1 group and NC group, respectively.

**Quantitative real-time PCR.** To quantitatively determine the mRNA expression level of HMGB1 in GAC cell lines (SGC-7901 and AGS), real-time PCR was performed. Total RNA was extracted from each clone using TRIzol according to the manufacturer’s protocol. Reverse transcription was carried out using M-MLV and cDNA amplification was performed using the SYBR Green Master Mix kit according to the manufacturer’s guidelines. The HMGB1 gene was amplified using a specific oligonucleotide primer and the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous control. The PCR primer sequences were as follows: HMGB1, 5’-ATATGGCAAGGCCGGACAG-3’ and 5’-AGGCCAGATGTCTCTCTTT-3’; GAPDH, 5’-CAAGGAATTTGGCTACAGCA-3’ and 5’-AGGCCAGGATGTTCTCCTTT-3’. Three separate experiments were performed for each clone.

**Western blot assay.** GAC cell lines (SGC-7901 and AGS) were harvested and extracted using lysis buffer (Tris-HCl, SDS, mercaptoethanol and glycerol). Cell extracts were boiled for 5 min in loading buffer, and then an equal amount of cell extracts was separated on 15% SDS-PAGE gels. Separated protein bands were transferred onto polyvinylidene fluoride (PVDF) membranes, which were subsequently blocked in 5% skim milk powder. Primary antibodies against HMGB1, NF-κB p65, PCNA and MMP-9 were diluted according to the manufacturer’s instructions and incubated overnight at 4°C. Subsequently, horseradish peroxidase–linked secondary antibodies were added at a dilution of 1:1,000 and incubated at room temperature for 2 h. The membranes were washed 3 times with PBS, and the immunoreactive bands were visualized using the ECL Plus kit according to the manufacturer’s instructions. The relative protein levels in different cell lines were normalized to the concentration of GAPDH. Three separate experiments were performed for each clone.

**Cell proliferation assay.** Cell proliferation was analyzed using the MTT assay. Briefly, cells infected with KiSS1 virus were incubated in 96-well-plates at a density of 1x10⁴ cells per well with DMEM medium supplemented with 10% FBS. Cells were treated with 20 µl of MTT dye at 0, 24, 48 and 72 h, and subsequently incubated with 150 µl of DMSO for 5 min. The color reaction was measured at 570 nm using an Enzyme Immunoassay Analyzer (Bio-Rad, Hercules, CA, USA). The proliferation activity was calculated for each clone.

**Wound-healing assay.** GAC cells (SGC-7901 and AGS) were plated in each well of a 6-well culture plate and allowed to grow to 90% confluence. Treatment with Lv-shHMGB1 was then performed. The next day, a wound was created using a micropipette tip. The migration of cells towards the wound was monitored daily, and images were captured at time intervals of 24 h.

**3D-Matrigel assay.** Cells were cultured on growth factor-depleted Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) using the overlay method. Briefly, 4-chambered Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY, USA) or 24-well plates were coated with Matrigel. Cells (2.0x10⁴ cells/ml for SGC-7901 and AGS) suspended in 2.5% Matrigel solution were added on coated chamber slides and allowed to grow up to 10 days.

**Subcutaneous tumor model and gene therapy.** Six-week-old female immune-deficient nude mice (BALB/c-nu) were bred at the laboratory animal facility (Institute of Chinese Academy of Sciences, Shanghai, China), and were housed individually in microisolator ventilated cages with free access to water and food. All experimental procedures were performed according to the regulations and internal biosafety and bioethics guidelines of Shanghai Jiaotong University and the Shanghai Municipal Science and Technology Commission. Two mice were injected subcutaneously with 1x10⁶ GAC cells in 50 µl of PBS pre-mixed with an equal volume of Matrigel matrix (Becton-Dickinson). Mice were monitored daily and developed a subcutaneous tumor. When the tumor size reached ~5 mm in length, they were surgically removed, cut into 1-2 mm³ pieces, and re-seeded individually into other mice. When tumor size reached ~5 mm in length, the mice were randomly assigned as NC group and Lv-shHMGB1 group. In Lv-shHMGB1 group, 15 µl of lentivirus was injected into subcutaneous tumors using a multi-site injection format. Injections were repeated every other day after initial treatment. The tumor volume every three days was measured with a caliper, using the formula: volume = (length x width)² / 2.

**Statistical analysis.** SPSS 20.0 was used for the statistical analysis. Kruskal-Wallis H test and χ² test were used to analyze the expression rate in all groups. One-way analysis of variance (ANOVA) was used to analyze the differences between groups. The LSD method of multiple comparisons was applied when the probability for ANOVA was statistically significant. Statistical significance was set at P<0.05.

**Results**

The expression of HMGB1 in gastric cancer tissues. The expression of HMGB1 protein was evaluated using IHC staining in gastric cancer tissues. As shown in Fig. 1, different
Figure 1. The expression of HMGB1 protein in GAC tissues (magnification, x200). GAC tissues were immunohistochemically stained with an anti-HMGB1 antibody and classified as positive expression (A) and negative expression (B). Adjacent non-cancer tissues were immunohistochemically stained with an anti-HMGB1 antibody and classified as positive expression (C) and negative expression (D). Positive immunostaining of HMGB1 was mainly localized in the nucleus and cytoplasm of tumor and tissue cells. Scale bars (A-D), 75 µm.

Figure 2. The infection efficiency of Lv-shHMGB1 in GAC cells. Lentiviruses of different MOI (10, 20 and 50) were transfected into GAC cell lines (SGC-7901 and AGS), and the infection efficiency was observed by fluorescence microscopy, Lv-shHMGB1 (MOI 50) was the highest, reaching >90%.
levels of positive expression of HMGB1 protein were detected in GAC and ANCT tissues. Positive HMGB1 immunostaining was mainly localized in the nucleus of gastric cancer tissue cells. According to the HMGB1 immunoreactive intensity, the positive expression of HMGB1 in gastric cancer tissues was significantly increased compared with that of HMGB1 in ANCT (P<0.001) (Table I).

Correlation of HMGB1 expression with clinicopathological parameters. According to the HMGB1 immunoreactive intensity, 51 (57.95%) patients were classified as low HMGB1 group, and 37 (42.05%) were classified as high HMGB1 group. We then analyzed the association between HMGB1 expression and the clinicopathological data of the patients with gastric cancer. As summarized in Table II, we found that high expression of HMGB1 was closely correlated with the metastatic lymph node of GAC (P=0.018), but did not correlate to the other clinicopathological factors including age, gender, tumor size, cancer TNM stage, and depth of invasion (each P>0.05).

The effect of HMGB1 knockdown on NF-κB expression. First, lentiviruses of different multiplicity of infection (MOI) were used to infect GAC cell lines (SGC-7901 and AGS), and the infection efficiency was observed by fluorescence microscopy. As shown in Fig. 2, the infection efficiency of Lv-shHMGB1 (MOI 50) was the highest, reaching >90%. After Lv-shHMGB1 (MOI 50) transfection into GAC cells (SGC-7901 and AGS) for 24 h, the expression levels of HMGB1 mRNA (Fig. 3A and B) and protein (Fig. 3C and D) and NF-κB p65 protein (Fig. 3C and D) were detected by real-time PCR and western blot assays, indicating clear inhibition of HMGB1 and NF-κB p65 expression in Lv-shHMGB1 group compared with the NC group (each **P<0.01).

Table I. The expression of HMGB1 protein in GAC tissues.

<table>
<thead>
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<th>Variables</th>
<th>Group</th>
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<th>-</th>
<th>+</th>
<th>++</th>
<th>+++</th>
<th>Positive rate (%)</th>
<th>χ²</th>
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<td>10</td>
<td>41</td>
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<td>ANCT</td>
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<td>17</td>
<td>2</td>
<td>70.5</td>
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GC, gastric adenocarcinoma; ANCT, adjacent non-cancer tissue.
of HMGB1 markedly diminished the proliferative activities of GAC cells in a time-dependent manner compared to the NC group (Fig. 4A and C). In addition, the expression level of PCNA protein, examined by western blot assay (Fig. 4B and D), was found significantly downregulated in Lv-shHMGB1 group compared with the NC group.

The effect of HMGB1 knockdown on cell migration and invasion. To determine the effect of HMGB1 knockdown on cell migration and invasion, wound-healing and 3D-Matrigel assays were performed. The results indicated that, the migration capabilities of GAC cells (SGC-7901 and AGS) in Lv-shHMGB1 group were markedly weakened at 24 h compared with those in NC group (Fig. 5A and B). GAC cells grew very slowly in 3D environments and therefore were allowed to grow up to 10 days. In Matrigel, the cells formed small mostly round masses, and Lv-shHMGB1 treatment induced no consistent changes in cell size and morphology of the cells. In contrast, the average size of GAC masses was much smaller in Lv-shHMGB1 group (25.50±7.90 µm) than that of NC group (45.30±6.41 µm) at day 10 (P<0.01, Fig. 5C). In addition, the expression level of MMP-9 protein, examined by western blot assay (Fig. 5D), was found significantly downregulated in Lv-shHMGB1 group compared with the NC group.

The effect of HMGB1 knockdown on xenograft tumor growth. Our in vitro experiments demonstrated the inhibitory effect of HMGB1 knockdown on tumor growth of GAC cells. Therefore, it is necessary to further investigate the effect of HMGB1 knockdown on xenograft tumor growth in vivo. The mean volume of tumors in the experimental mice before treatment was 58.68±28.22 mm³. During the whole tumor growth period, the tumor growth activity was measured. The tumors treated with Lv-shHMGB1 grew substantially slower compared to the NC group (Fig. 6A and B). When the tumors were harvested,
the average weight and volume of the tumors in Lv-shHMGB1 group were significantly smaller than those of the NC group (Fig. 6C and D), suggesting that the knockdown of HMGB1 suppressed the growth of GAC cells.

**Discussion**

HMGB1 plays an important role in a number of clinical conditions, such as autoimmunity, cardiovascular disease and...
cancer. HMGB1 promotes proliferation and invasion of lung cancer by activation of the Erk1/2 and p38MAPK pathways (22). HMGB1 is correlated with angiogenesis, acts as a key regulator in the progression of carcinoma, and serves as a potential diagnostic and therapeutic target (23,24). HMGB1 is overexpressed in tumor cells and promotes activity of regulatory T cells in patients with head and neck cancer (25). Expression of HMGB1 is also significantly associated with malignancy and clinical stage of T-cell lymphomas, and may be an important biomarker for the development and progression of T-cell lymphoma (26). The expression of HMGB1 is closely correlated with pathological grade and distant metastases of liver cancer, and knockdown of HMGB1 inhibits liver cancer growth and metastasis, suggesting that HMGB1 may represent a potential therapeutic target for the aggressiveness of this malignancy (27).

Importantly, the expression of HMGB1 is increased in gastric cancer with the intestinal type compared to that in the diffuse type, and is linked to the degree of macrophage infiltration in the tumor microenvironment (28). In the present study, we also found that the positive expression of HMGB1 was significantly increased in the nucleus of gastric cancer tissues compared with the adjacent non-cancer tissues. Similar with other studies (10,26-28), HMGB1 was found correlated with metastatic lymph nodes in gastric cancer, suggesting that nuclear accumulation of HMGB1 might be involved in the development and progression.

Furthermore, the focus of research should be on the function of HMGB1 in cancer. In this study, a loss of function experiment was performed to clarify the function of HMGB1 in GAC cells, showing that knockdown of HMGB1 gene by shRNA suppressed cell proliferative activities and invasive potential, and slowed xenograft tumor growth. Taken into account the correlation of HMGB1 with lymph node metastasis of GAC, our findings indicated that HMGB1 might play an important role in promoting tumorigenesis of GAC. Increased expression of HMGB1 is also associated with distant metastases of liver cancer, and knockdown of HMGB1 inhibits liver cancer growth and metastasis (27). The HMGB1/RAGE inflammatory pathway directly promotes pancreatic tumor growth by regulating mitochondrial bioenergetics (29). Importantly, ethyl pyruvate, a potent inhibitor of HMGB1 has been shown to inhibit tumor growth and metastasis and have a therapeutic role in the treatment of cancer in conjunction with other therapeutic agents (30,31). Though few studies indicate HMGB1 not to be the principal mediator of inflammation in malignant epidermal tumors (32), most studies, including ours, support HMGB1 functions as a tumor-promoting gene, and may serve as a potential therapeutic target for treatment of cancer.

PCNA is a nuclear protein that is expressed in proliferating cells and may be required for maintaining cell proliferation, used as a marker for cell proliferation of gastric cancer (33). MMP-9 is thought to be a key enzyme involved in the degradation of type IV collagen and high level of MMP-9 in tissues is associated with tumor growth and invasion (34). It has been reported that blockade of NF-κB pathway by RNAi inhibits growth and metastasis of malignant tumors via regulation of PCNA and MMP-9 expression (35). In our study, it was found that knockdown of HMGB1 downregulated the expression of NF-κB, PCNA and MMP-9 in GAC cells, suggesting that targeted blockade of HMGB1 pathway might inhibit growth
and metastasis of GAC cells through NF-κB-mediated regulation of PCNA and MMP-9 expression.

In conclusion, these findings suggest that increased expression of HMGB1 is associated with tumor metastasis of GAC, and the knockdown of HMGB1 suppresses growth and invasion of GAC cells through the NF-κB pathway in vitro and in vivo, thus HMGB1 may serve as a potential therapeutic target for GAC.

Acknowledgements

This study was supported by National Nature Science Foundation of China (nos. 81302093 and 81272752).

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