# Extracellular high mobility group box chromosomal protein 1 promotes drug resistance by increasing the expression of P-glycoprotein expression in gastric adenocarcinoma cells

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**Abstract.** It has previously been reported that high mobility group box chromosomal protein 1 (HMGB1) is overexpressed in the majority of gastric adenocarcinoma cell types, and that HMGB1 can be released into the extracellular matrix from stressed or necrotic cancer cells. HMGB1 is considered to promote cell proliferation and invasion in gastric adenocarcinoma cells. Furthermore, in a number of cancer cell types, HMGB1 has been reported to promote autophagy and inhibit anticancer drug-induced apoptosis, which has been identified as an important mechanism in the development of multidrug resistance (MDR). However, there have been no studies on the effects of HMGB1 on expression of the MDR-related transporter proteins in gastric adenocarcinoma. In the present study, extracellular HMGB1 increased the expression levels of P-glycoprotein (P-gp) at the pre-transcriptional and post-transcriptional levels in the human gastric adenocarcinoma cell lines, SGC7901, MKN28 and AGS, as detected by quantitative polymerase chain reaction and western blot assays. MTT and apoptosis assays were also performed and it was demonstrated that extracellular HMGB1 subsequently enhanced resistance to the P-gp-related drugs, adriamycin and vincristine. In brief, this study demonstrated that extracellular HMGB1 may promote drug resistance to adriamycin and vincristine by upregulating P-gp in human gastric adenocarcinoma cells.

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## Introduction

The high mobility-group box-1 protein (HMGB1), belonging to a 'group of chromatin-associated proteins with high acidic and basic amino acid contents', exists in the nucleus of almost all eukaryotic cells (1,2). As a nuclear protein, it can act intracellularly as a nuclear DNA-binding protein or extracellularly as a cytokine-like signaling molecule (3). Extracellular HMGB1 can be actively secreted via macrophage secretion, and passively released from stressed and necrotic cells (4). Studies have shown that the dysregulation of HMGB1 may be linked to numerous malignancies, for example pancreatic, breast, colon and gastric cancer (5,6).

Gastric carcinoma is the fourth most common malignant disease and the second leading cause of malignant mortality worldwide, and chemotherapy is considered to be one of the most important treatments for advanced gastric cancer (7). However, the therapeutic effects of chemotherapy for gastric cancer are compromised by the existence of multidrug resistance (MDR), which has been recognized as a major barrier in anticancer therapy (8). Failure of drug-induced apoptosis and reduced drug accumulation are recognized as two major mechanisms for the development of MDR in cancer (9). As an extracellular damage-associated molecular pattern or necrotic marker (10), HMGB1 is overexpressed in the majority of gastric adenocarcinoma cells (11). Additionally, HMGB1 acts as a signaling-like protein following extracellular release from stressed and necrotic tumor cells (12), which may inhibit apoptosis and help tumor cells escape cytotoxicity in a number of cancer cells, by activating the receptor for advanced glycation end products in tumor cells (13).

In previous decades, various transporter proteins inside gastric cancer cells have been reported to increase chemotherapy resistance. Of these extensively studied proteins, P-glycoprotein (P-gp) has gained considerable attention (14,15). There are no reports focusing on the relationship between extracellular HMGB1 and chemotherapy

resistance-related transporter proteins in gastric adenocarcinoma cells.

In the present study, the effect of extracellular release of HMGB1 by tumor cells on the expression of the chemotherapy resistance-related transporter proteins was analyzed in gastric adenocarcinoma cells. In addition, the effects of HMGB1 on resistance to anticancer drugs was determined.

## Materials and methods

*Cell culture*. The human gastric adenocarcinoma cell lines, SGC7901, MKN28 and AGS, were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RMPI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA, USA) at 37˚C in a humidified atmosphere and 5% $CO_2$. The medium was routinely changed 3 days after seeding.

*Drug sensitivity assay in vitro*. HMGB1 was purchased from HMGBiotech s.r.l. (Milan, Italy). The effects on the resistance to chemotherapeutic drugs in SGC7901, MKN28 and AGS cells were determined by MTT assay (Sigma-Aldrich, St. Louis, MO, USA). SGC7901, MKN28 and AGS cells were all divided into three groups: Control group with culture medium only; cells treated with HMGB1 at 50 ng/ml for 48 h prior to drug sensitivity assay and cells treated with HMGB1 for 48 h at 25 ng/ml prior to drug sensitivity assay. Cells in various groups were seeded into 96 well-plates at a density of 4,000 cells per well and incubated to attach overnight. Following adhesion, cells were cultured for 72 h in the presence or absence of various concentrations of four anticancer drugs, adriamycin (ADM; Qilu Pharmaceutical Co., Ltd., Jinan, China), vinicristine (VCR; Qilu Pharmaceutical Co., Ltd.), 5-fluorouracil (5-Fu) and cisplatin (cDDP; Sigma-Aldrich), in 100 $\mu$l medium. Next, 20 $\mu$l MTT was added to each well and cells were incubated for another 4 h. Following removal of the supernatants from each well, 150 $\mu$l dimethyl sulfoxide (Sigma-Aldrich) was added to each well to dissolve any crystals. The absorption of each well was detected at 490 nm by Multiskan Ascent (Thermo Fisher Scientific). The cell viability of each well was calculated by the standard formula for MTT assay, and the IC50 values for the drugs in each group of each cell line were examined.

*Apoptosis assay by flow cytometry*. To detect the effects of extracellular HMGB1 on the apoptosis of gastric cancer cells induced by chemotherapy agents, ADM and VCR were added to AGS cells divided into the indicated groups. After 24 h, cells were trypsinized and washed twice with cold phosphate-buffered saline. Next, cells were resuspended in binding buffer and FITC-Annexin V and propidium iodide (KenGen, Nanjing, China) were added to fixed cells. The mixture was left to react for 30 min in the dark at room temperature and FACSort flow cytometery was used to determine the fluorescence of cells.

*RNA isolation and quantitative polymerase chain reaction (qPCR) amplification*. SGC7901, MKN28 and AGS cells were seeded into six-well plates and treated with the indicated concentrations of HMGB1 (50 and 25 ng/ml). The control groups were treated with culture medium only. Following incubation for 48 h, total cell RNA of each well was extracted using TRIzol reagent (Takara Bio, Inc., Shiga, Japan). Reverse transcription was performed using M-MLV (Takara Bio, Inc.) and cDNA in each sample was amplified using an RNA PCR kit (Takara Bio, Inc.). qPCR was performed according to the manufacturer's instructions. MDR1 was amplified using specific primers (Wuhan Biobuffer Biology, China) and the housekeeping gene, β-actin (Wuhan Biobuffer Biology), was used as an endogenous control. The RT-PCR primer sequences for P-gp were: Forward, 5'-TGATTGCATTTGGAGGACAA-3' and reverse, 5'-CCAGAA GGCCAGAGCATAAG-3'. The primer sequences for β-actin were: Forward, 5'-AGCGAGCATCCCCCAAAGTT-3' and reverse, 5'-GGGCACGAAGGCTCATCATT-3'. All results were analyzed using the comparative CT value method.

*Western blot analysis*. SGC7901, MKN28 and AGS cells were seeded into six-well plates and treated with the indicated HMGB1 concentrations (50 and 25 ng/ml) for 48 h. Control groups were set with culture medium only. Following the collection of cells of each group from six-well plates, total protein was extracted using a protein extract kit (Beyotime Institute of Biotechnology, Shanghai, China), according to the manufacturer's instructions, and samples were separated by 10-12% SDS-PAGE and analyzed using a rabbit primary antibody against P-gp (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The expression of β-actin (Sigma-Aldrich) was measured to normalize the total protein loaded in each sample. The resulting immunoblots were visualized using an enhanced chemiluminescence substrate system (Beyotime Institute of Biotechnology).

*Statistical analysis*. All experiments were performed at least three times. Values were presented as the mean ± standard deviation and the Student's t-test was used for numerical data analysis. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Extracellular HMGB1 significantly increases drug resistance to multiple chemotherapy drugs in SGC7901, MKN28 and AGS cell lines*. Diverse anticancer drugs have been used to treat gastric carcinoma patients (16) but the underlying drug resistance contributes to the limited benefit of these regimens in advanced gastric adenocarcinoma (8). To determine the effect of extracellular HMGB1 on the resistance to anticancer drugs in SGC7901, MKN28 and AGS cells, the chemosensitivity to ADM, VCR, cDDP and 5-Fu was investigated in these three cell lines. MTT assay was used to determine the chemosensitivity of various anticancer drugs of different groups in the three gastric adenocarcinoma cell lines.

As shown in Fig. 1, the IC50 values for ADM and VCR in HMGB1-treated gastric adenocarcinoma cells were significantly increased compared with the control groups ($P<0.05$). However, no effects of extracellular HMGB1 on

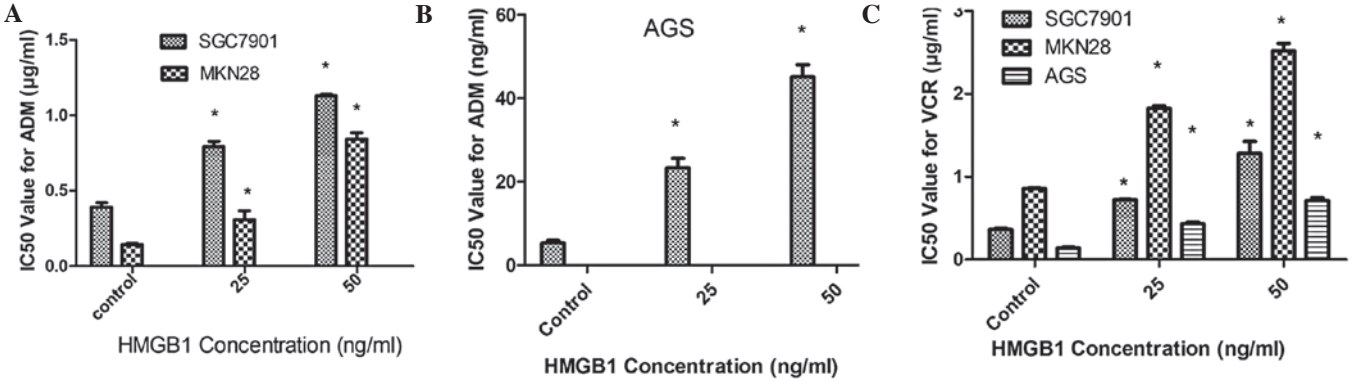


Figure 1. Following culture with various concentrations of HMGB1 for 48 h, cells were treated with different concentrations of anticancer drugs for 72 h and the MTT assay was used to determine the $IC_{50}$ values. (A and B) MTT assay revealed that extracellular HMGB1 significantly enhanced drug resistance to ADM. (C) Extracellular HMGB1 enhanced the $IC_{50}$ value of VCR in gastric adenocarcinoma cells. *P<0.05, vs. control. HMGB 1, high mobility group box chromosomal protein 1; ADM, adriamycin; VCR, vinicristine.

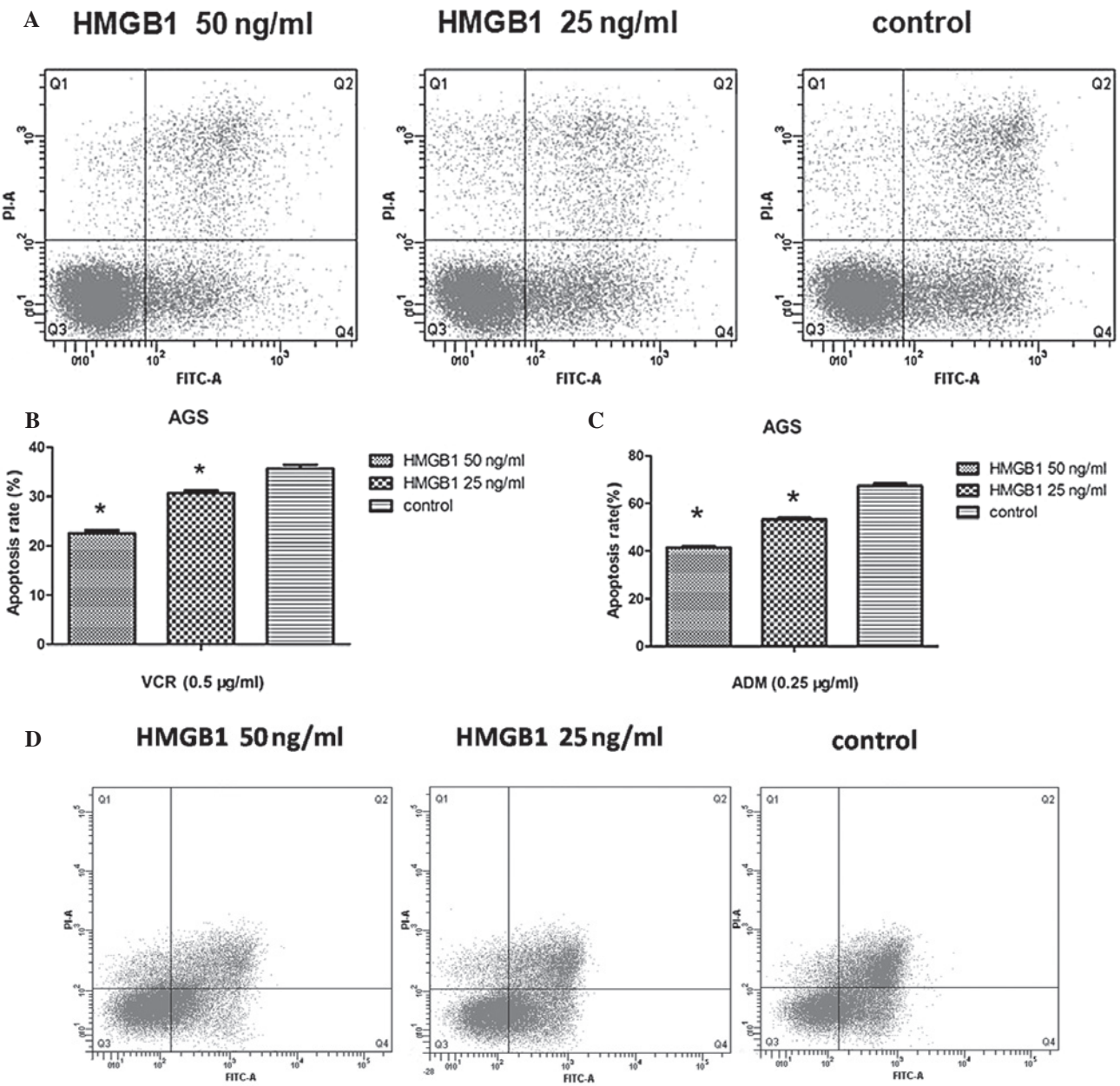


Figure 2. Apoptosis rates in the AGS cell line were detected by flow cytometry. (A and B) Extracellular HMGB1 significantly inhibited VCR-induced apoptosis (0.5 $\mu$g/ml). (C and D) Extracellular HMGB1 also decreased ADM-induced apoptosis (0.25 $\mu$g/ml). *P<0.05, vs. control. HMGB 1, high mobility group box chromosomal protein 1; ADM, adriamycin; VCR, vinicristine.

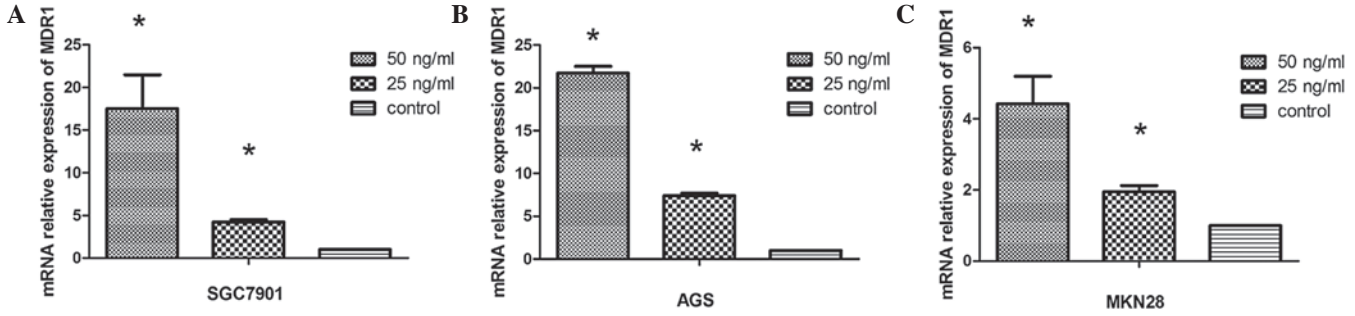


Figure 3. Extracellular HMGB1 enhances the expression of the MDR1 (P-glycoprotein) gene in SGC7901, MKN28 and AGS cells. The expression of MDR1 in three groups was detected by reverse transcription polymerase chain reaction. (A-C) Comparative computed tomography was used to determine the effect of various concentrations of HMGB1 on the expression of MDR1 in gastric adenocarcinoma cells. *P<0.05, vs. control. HMGB 1, high mobility group box chromosomal protein 1; MDR1, multidrug resistance 1; ADM, adriamycin; VCR, vinicristine.

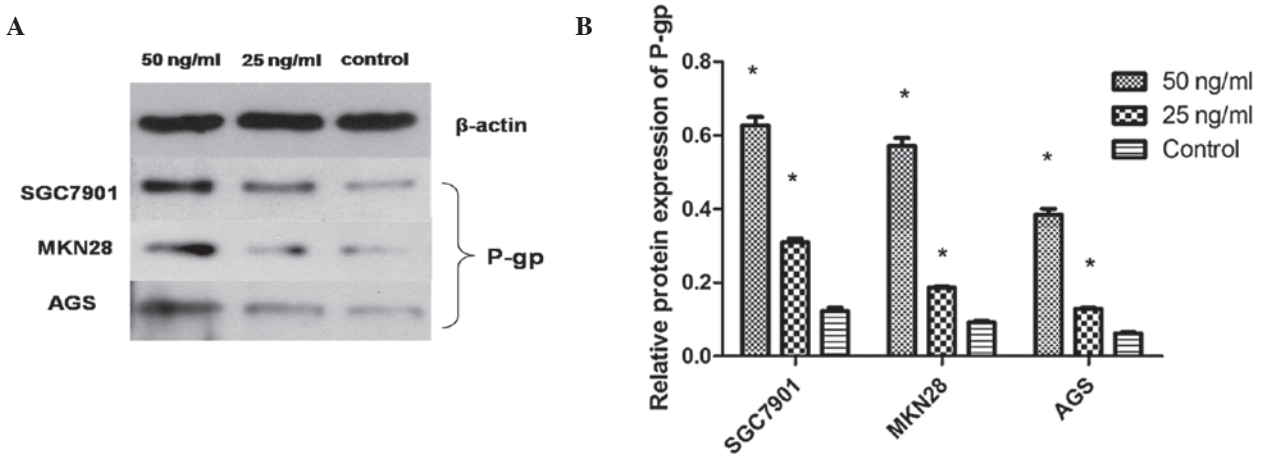


Figure 4. Detection of P-gp expression by western blot analysis. (A) Electrophoregram and (B) bar graph demonstrating that extracellular HMGB1 significantly increases the expression of P-gp in gastric adenocarcinoma cells. *P<0.05, vs. control. HMGB 1, high mobility group box chromosomal protein 1; P-gp, P-glycoprotein.

the chemosensitivity to 5-Fu and cDDP were observed. Furthermore, the apoptosis assay showed that extracellular HMGB1 could significantly decrease the apoptosis rate induced by ADM and VCR in gastric adenocarcinoma cells (Fig. 2). Taken together, the results indicate that extracellular HMGB1 increases cell resistance to ADM and VCR.

*Extracellular HMGB1 increases P-gp gene and protein expression levels in SGC7901, MKN28 and AGS cell lines.* The results of MTT assay and apoptosis analysis indicated that extracellular HMGB1 increases the expression of several MDR-related transporter proteins. The most widely known chemotherapeutic resistance-associated transporter protein, P-gp, is described as playing an important role in the development of MDR in gastric adenocarcinoma. There are several mechanisms involved in the effects of P-gp during the development of MDR, including decreased drug accumulation and increased drug influx and inactivation. P-gp was the first adenosine triphosphate (ATP)-binding cassette family protein identified by researchers and is the product of the human MDR1 gene localized to chromosome 7q21 (17). In gastric adenocarcinoma, the expression of P-gp has a marked effect on the pharmacokinetics of numerous anticancer drugs, including ADM and VCR. P-gp can reduce the intracellular drug concentration by binding to the drug and acting as an ATP-dependent efflux pump, and overexpression of P-gp may enhance resistance to ADM and VCR (18). Thus, the effect of extracellular HMGB1 on the expression of P-gp in SGC7901, MKN28 and AGS cells was investigated.

As shown in Fig. 3, qPCR results revealed that extracellular HMGB1 significantly upregulated expression of MDR1 in a concentration-dependent manner. Additionally, western blot analysis demonstrated that extracellular HMGB1 consistently enhanced the P-gp protein expression levels, as shown in Fig. 4.

## Discussion

Overexpression of HMGB1 exists in the majority of tumor cell types, including gastric adenocarcinoma and colorectal and breast cancer (19), and can be passively released extracellularly by diffusion from the leaky membranes of unscheduled necrotic tumor cells (20). Necrosis is a common phenotype in advanced solid cancers and can be induced by certain adjuvant therapies for cancer, including chemotherapy and radiotherapy (21). The hypothesis that necrotic cancer cells can deliver signals to protect remaining cancer cells has been investigated over sixty years (22), however, the mechanisms

behind this notion are complex. In previous studies, HMGB1 has been considered to regulate the balance of tumor cell apoptosis and autophagy in gastric adenocarcinoma cells, and thus enable tumor cells to escape the cytotoxic effects of numerous anticancer drugs. In addition, the results of clinical research have shown that higher levels of extracellular HMGB1 may lead to a poorer prognosis in gastric adenocarcinoma (23). Thus, extracellular HMGB1 release from necrotic or stressed cancer cells may play a critical role in the development of chemotherapy resistance.

In the present study, MTT and apoptosis assay results demonstrated that extracellular HMGB1 may enhance the resistance of gastric adenocarcinoma cells to anticancer drugs, which indicates a direct role of extracellular HMGB1 in the development of chemoresistance. Therefore, the effects of extracellular HMGB1 on the expression of a well-known MDR-related protein was analyzed. The results of qPCR and western blot analysis showed that extracellular HMGB1 significantly increases the expression of P-gp in gastric adenocarcinoma cells.

In conclusion, the present study has demonstrated for the first time that extracellular HMGB1 may significantly increase the expression of P-gp in human gastric adenocarcinoma cells, thus increasing the resistance to anticancer drugs and promoting MDR. Although additional mechanisms behind this biological phenomenon require further investigation, these results will help to further understand MDR in gastric adenocarcinoma and to provide new strategies to overcome MDR. In addition, the chemotherapeutic effects of anticancer drugs may be predicted and proper regimens may be selected, by investigating the level of extracellular HMGB1 in human serum or the tumor microenvironment.

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