Prognostic Significance of High Mobility Group Box 1 (HMGB1) Expression in Patients with Colorectal Cancer

MASAMI UEDA[1,2], YUSUKE TAKAHASHI[2], YOSHIAKI SHINDEN[1], SHOTARO SAKIMURA[1], HIDENARI HIRATA[1], RYUTARO UCHI[1], YUKI TAKANO[1], JUNJI KURASHIGE[1], TOMOHIRO IGUCHI[1], HIDETOSHI EGUCHI[1], KEISHI SUGIMACHI[1], HIROFUMI YAMAMOTO[2], YUICHIRO DOKI[2], MASAKI MORI[2] and KOSHI MIMORI[1]

[1]*Department of Surgery, Kyushu University Beppu Hospital, Beppu, Japan;*
[2]*Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan*

Abstract. *Background: High mobility group 1 (HMGB1) is a highly conserved non-histone nucleosomal protein in mammals. We investigated the clinical significance of HMGB1 expression in colorectal cancer (CRC). Patients and Methods: The expression of HMGB1 mRNA in 140 tumor and normal tissues from CRC patients was examined by quantitative real-time polymerase chain reaction (PCR). We immunohistochemically investigated HMGB1 expression in tumor and metastatic lymph nodes in CRC. Results: HMGB1 expression was significantly higher in tumor than in normal tissues. High HMGB1 expression was associated with larger tumor volumes, higher rates of lymphatic invasion, more frequent lymph node metastases and poorer prognoses for overall survival. Multivariate analyses showed that HMGB1 expression was an independent prognostic indicator of overall survival. Immunocytochemical analysis revealed that HMGB1 was overexpressed in both CRC tissues and regional lymph node metastases. Conclusion: Investigating HMGB1 expression may be a predictor of postoperative lymph node metastasis and prognosis in CRC.*

High morbidity group box 1 (HMGB1) is a non-histone nucleosomal protein that is widely expressed and highly conserved in mammals. HMGB1 localizes to the nucleus and nuclear HMGB1 interacts with various transcription factors, such as TATA-binding protein (TBP) and p53 (1). Additionally, HMGB1 can be released into the extracellular matrix through 2 mechanisms: passive release by necrotic and damaged cells (2) or secretion by activated monocytes, macrophages and pituicytes derived from an environment containing exogenous bacterial products or endogenous pro-inflammatory cytokines (3-5). HMGB1 plays a major role in many physiological and pathological conditions, including arthritis (6), cardiovascular disease (7), inflammation (8), ischemia (9), meningitis (10) and sepsis (11).

In cancer, HMGB1 expression has been shown to be associated with almost every tumor type, particularly epithelial neoplasms (12-16). Colorectal cancer (CRC) is one of the most common types of cancer worldwide and its invasive and metastatic properties result in a high rate of cancer-related deaths (17). Metastasis is responsible for as much as 90% of cancer-associated mortality; therefore, identification and regulation of genes responsible for metastasis is essential to improve prognoses in patients with CRC. Earlier reports have revealed that CRC tumor tissues contain higher HMGB1 levels than non-cancerous tissues, as measured by immunohistochemical staining (18).

In the current study, we investigated the clinical significance of *HMGB1* expression in CRC tumor tissues, particularly in distant metastases. Furthermore, we examined the HMGB1 expression in tumor tissues and regional lymph node metastases by immunohistochemical analysis.

Materials and Methods

Clinical samples. A total of 140 CRC samples and paired non-cancerous tissues were obtained during surgery. These samples were used in accordance with the ethical guidelines of Kyushu University after obtaining written informed consent from all patients. All patients underwent resection of the primary tumor at Kyushu University Hospital and affiliated hospitals between 1992 and 2002. All patients were clearly identified as having CRC based on clinicopathological findings, including tumor size and depth, lymphatic invasion, lymph node metastasis, vascular invasion, liver metastasis, peritoneal dissemination, distant metastasis, as well as clinical and pathological records. The median follow-up was 2.93 years. Resected (T) and paired (N) tissues were immediately cut and stored in RNAlater (Ambion, Austin, TX, USA), frozen in liquid

Correspondence to: Prof. Koshi Mimori, MD, Ph.D., Department of Surgery, Kyushu University, Beppu Hospital, 4546 Tsurumihara, Beppu, Oita, 874-0838, Japan. Tel: +81 977271650, Fax: +81 977271651, e-mail: mueda@beppu.kyushu-u.ac.jp

Key Words: HMGB1, colorectal cancer, lymph node metastasis.

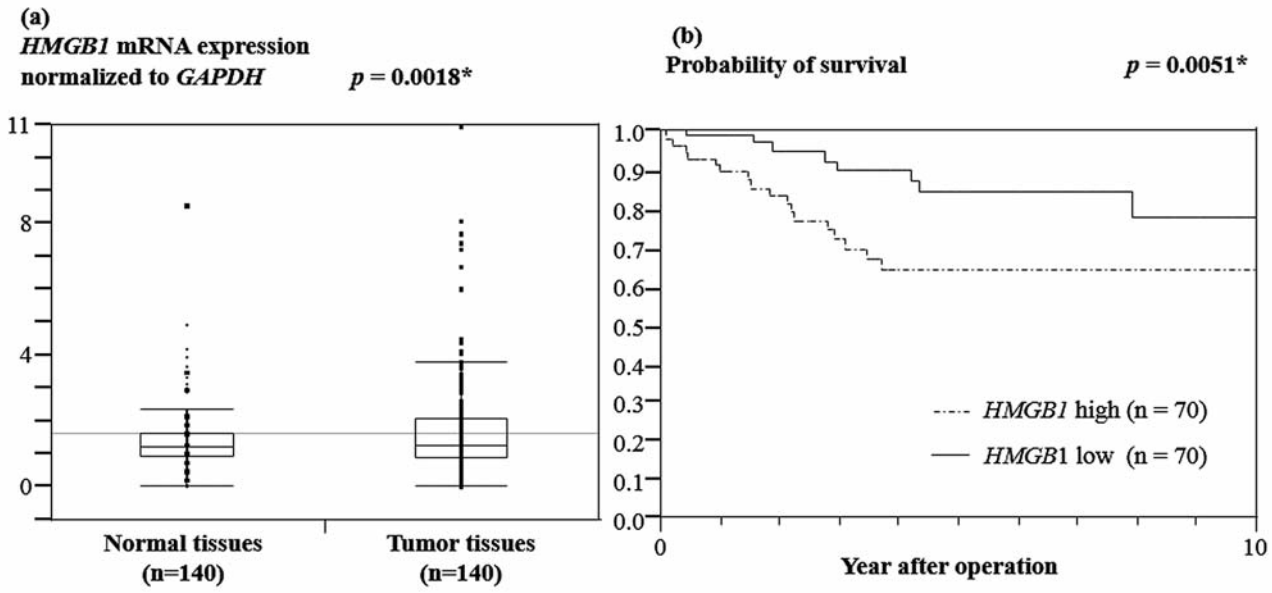

Figure 1. *Clinical significance of HMGB1 mRNA expression. (A) HMGB1 expression levels as measured by quantitative real-time PCR in 140 normal tissues and tumor tissues. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; (B) Kaplan-Meier overall survival curves according to HMGB1 levels. The overall survivals of patients with high HMGB1 expression (n=70) was significantly higher than that of patients with low expression (n=70); log-rank test, p=0.0051. *p<0.05.*

nitrogen and kept at –80˚C until RNA extraction. RNA was extracted using ISOGEN (NipponGene, city, Japan) according to the manufacturer's protocol.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). Gene-specific oligonucleotide primers were designed for qRT-PCR. The following primers were used: *HMGB1*, 5'-CATTGAGCTCCATAGAGACAGC-3' (sense) and 5'-GGATCT CCTTTGCCCATGT-3' (antisense); and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 5'-TTGGTATCGTGGAAGGACTCA-3' (sense) and 5'-TGTCATCATATTTGGCAGGTT-3' (antisense). PCR amplification was performed in a LightCycler 480 instrument (Roche Applied Science, Basel, Switzerland) using a LightCycler 480 Probes Master kit (Roche Applied Science, address). Amplification conditions for the *HMGB1* mRNA consisted of initial denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 10 s, annealing at 62˚C (60˚C for other genes) for 10 s and elongation at 67˚C (65˚C for other genes) for 10 s. Melt curve analysis was performed to distinguish specific products from nonspecific products and primer dimers. The relative expression levels of these genes were obtained by normalizing the amount of mRNA to that of *GAPDH* mRNA as an endogenous control in each sample.

Histology and immunocytochemical analysis. Colon carcinoma tissues and metastatic lymph node tissues were surgically removed, embedded in paraffin and sectioned (5-mm sections). They were stained with hematoxylin and eosin (H&E) for histological analysis. Immunohistochemical analysis was applied to determine the localization of HMGB1. A polyclonal rabbit anti-HMGB1 antibody (1:100; Abcam, Cambridge, UK) was used as the primary antibody.

Table I. *Relationship between HMGB1 mRNA expression and clinicopathologic factors in 140 colorectal cancer patients.*

Factors		*HMGB1/GAPDH*		*p*-Value
		High expression (n=70)	Low expression (n=70)	
Age (years)	≤65	27	26	0.86
	65<	43	44	
Gender	Male	39	49	0.08
	Female	31	21	
Histology	Well moderate	64	66	0.74
	Others	6	4	
Size	<3 cm	8	20	0.01*
	3 cm≤	59	49	
Serosal invasion	Absent	40	51	0.051
	Present	30	19	
Lymph node metastasis	Absent	31	43	0.04*
	Present	39	27	
Lymphatic invasion	Absent	33	45	0.04*
	Present	37	25	
Venous invasion	Absent	51	59	0.09
	Present	19	11	
Liver metastasis	Absent	61	67	0.07
	Present	9	3	

*$p<0.05$.

Table II. *Univariate and multivariate analysis for overall survival (Cox proportional hazards regression model).*

	Univariate analysis			Multivariate analysis		
Factors	RR	95% CI	*p*-Value	RR	95% CI	*p*-Value
Tumor size (<30mm/30mm <)	2.11	1.15-5.28	0.01*	1.06	0.51-2.77	0.88
Serosal invasion (absent/present)	2.05	1.39-3.11	<0.001*	1.36	0.87-2.19	0.17
Lymphatic invasion (absent/present)	1.67	1.13-2.52	0.009*	1.05	0.65-1.72	0.82
Venous invasion (absent/present)	2.08	1.40-3.07	<0.001*	1.71	1.07-2.75	0.02*
Liver metastasis (absent/present)	3.75	2.42-5.61	<0.001*	2.94	1.69-5.12	<0.001*
Peritoneal metastasis (absent/present)	5.61	2.37-13.29	<0.001*	7.16	1.40-14.50	0.01*
Lymph node metastasis (absent/present)	2.22	1.45-3.68	<0.001*	1.26	0.74-2.25	0.39
HMGB1 expression (low/high)	1.66	1.11-2.60	0.01*	1.59	1.00-2.65	0.04*

RR, Rerative risk; CI, confidence interval; *$p<0.05$.

Statistical analysis. Data from qRT-PCR analyses were analyzed using the JMP 5 software (JMP, Cary, NC, USA). The relationships between *HMGB1* expression and clinicopathological factors were analyzed using the Student's *t*-tests, Chi-squared tests and analysis of variance (ANOVA). Overall survival (OS) curves were plotted using the Kaplan-Meier method measured from the day of surgery, while the log-rank test was applied for comparisons. All differences were statistically significant at the level of $p<0.05$. The relative multivariate significance of potential prognostic variables was also examined. The Cox proportional hazards regression was used to test the independent prognostic contribution of *HMGB1*.

Results

HMGB1 mRNA expression in 140 tumor tissues from CRC patients was examined by qRT-PCR to investigate the clinical significance of *HMGB1* in CRC. As a control, we measured *HMGB1* mRNA expression in normal tissues from the same patients. *HMGB1* expression was significantly higher in tumor than in normal tissues (Figure 1A). Additionally, we divided the 140 patients with CRC into a high-*HMGB1*-expression group (n=70) and a low-HMGB1-expression group (n=70) according to the median expression level in tumor tissues and analyzed clinicopathological factors in the high and low *HMGB1* mRNA expression groups (Table I). The high-*HMGB1*-expression group showed greater lymphatic invasion and lymph node metastasis than the low-*HMGB1*-expression group. Furthermore, the high-*HMGB1*-expression group exhibited significantly larger tumors than the low-*HMGB1*-expression group. With regard to OS, patients with high *HMGB1* expression had significantly poorer prognoses than those with low *HMGB1* expression ($p=0.0051$) (Figure 1B). Univariate and multivariate analyses showed that *HMGB1* mRNA expression was an independent prognostic indicator of OS in patients with CRC (relative risk, 1.59; $p=0.04$) (Table II).

To clarify the correlation of HMGB1 expression with CRC, HMGB1 protein levels were investigated in colon cancer tissues and lymph node metastases by immunohistochemical analysis. As shown in Figures 2 and 3, HMGB1 protein was highly expressed in cancer cells from both CRC tissues and corresponding metastatic lymph node tissues, suggesting that colon cancer cells expressing HMGB1 might lead to lymph node metastasis. Furthermore, high HMGB1 expression was present in the cytoplasm and nucleus of primary tumor tissues in case 1, while high HMGB1 expression was only in the cytoplasm in case 2.

Discussion

Our study revealed that *HMGB1* mRNA expression was significantly higher in CRC tissues than in noncancerous tissues. Several previous studies have supported the significance of HMGB1 in CRC, demonstrating that HMGB1 is overexpressed in CRC tissues. Xiang *et al*. first reported that colorectal adenocarcinoma tissues contain higher HMGB1 levels than corresponding noncancerous mucosa, as analyzed by tissue microarray (19). Moreover, elevated *HMGB1* mRNA levels have been detected in 40% of all colon carcinomas by using microarray analyses to establish expression profiles (18). In the current study, we presented definitive findings of *HMBG1* expression in CRC tissues and regional lymph node metastases using a much larger sample size than those described in previous reports.

Our study also, firstly, showed that *HMGB1* mRNA expression in CRC tissues was related to a poor prognosis and that high expression of *HMGB1* mRNA in CRC tissues was significantly associated with tumor volume, lymphatic invasion and lymph node metastasis. These data indicated that HMGB1 induced the progression, invasion and migration of cancer cells. To explore whether HMGB1 may be involved in lymphatic invasion and lymph node metastasis, we investigated the expression of HMGB1 in tumor tissues derived from primary tumors and lymph nodes by immunohistochemical analysis. We demonstrated that the

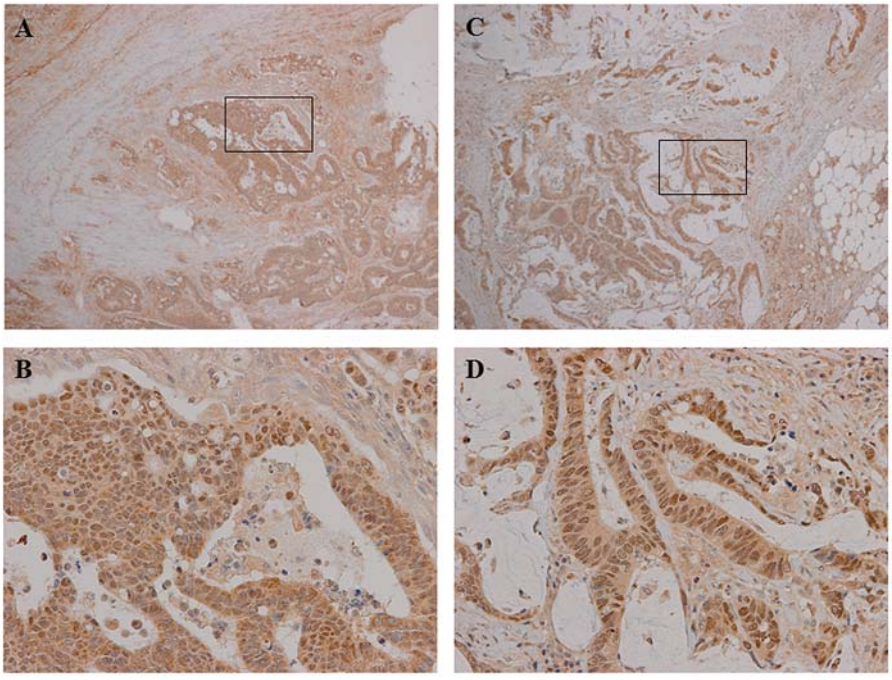

Figure 2. *Immunohistochemical staining in human clinical samples of CRC tissues and the corresponding lymph node metastasis tissues in case 1. (A) High expression of HMGB1 in both cytoplasm and nucleus of cancer cells in CRC tissues (original magnification ×40); (B) Larger magnifications (×200) of boxed region in (A); (C) High expression of HMGB1 in both cytoplasm and nucleus of cancer cells in the corresponding lymph node metastasis tissues (original magnification ×40); (D) Larger magnifications (×200) of boxed region in (C).*

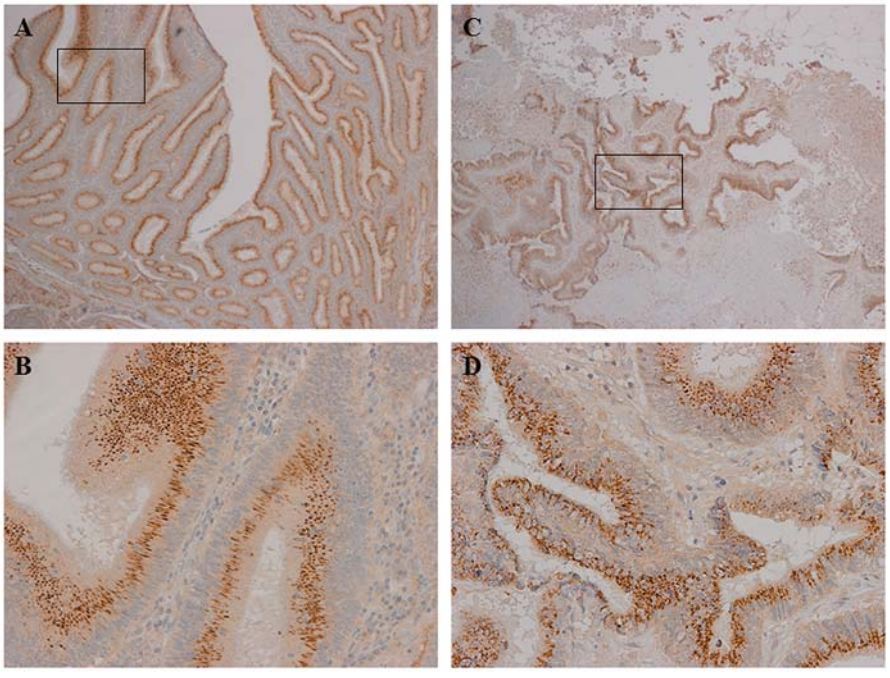

Figure 3. *Immunohistochemical staining in human clinical samples of CRC tissues and the corresponding lymph node metastasis tissues in case 2. (A) High expression of HMGB1 only in the cytoplasm of cancer cells in CRC tissues (original magnification ×40); (B) Larger magnifications (×200) of boxed region in (A); (C) High expression of HMGB1 only in the cytoplasm of cancer cells in the corresponding lymph node metastasis tissues (original magnification ×40); (D) Larger magnifications (×200) of boxed region in (C).*

level of HMGB1 expression in lymph node metastases was equivalent to that of corresponding primary tumors.

Moreover, our immunohistochemical analysis revealed that 20% of malignant cells expressed the HMGB1 protein in both the cytoplasm and nucleus; however, the remaining cancer cells showed HMGB1 expression only in the cytoplasm. In previous studies, HMGB1 has been shown to act as a tumor-promoting factor, performing multiple functions. Inside the cell, HMGB1 is a highly conserved chromosomal protein that acts as a DNA chaperone that has been known to enhance the activity of ftranscriptional activators and repressors by binding to transcription factors (20, 21). HMGB1 is also released into the extracellular space, where it binds to cell surface receptors, such as receptor for advanced glycation end-products [RAGE] and Toll-like receptor 4 [TLR4], to activate the downstream signaling pathways (nuclear factor κB [NF-κB], mitogen-activated protein kinase [MAPK] and phosphoinositol 3 kinase [PI3K]). Activation of these downstream pathways produces a functional response, leading to activation of cell adhesion and migration, promotion of cell proliferation and induction of angiogenesis (22-24). These previous studies support that HMGB1 protein exists in both the cytoplasm and nucleus of cancer cells, consistent with the results of our immunocytochemical analysis.

As described above, the expression of HMGB1 was correlated with various clinicopathological factors and could be a critical indicator of tumor aggressiveness and metastasis in primary CRC. In particular, it is possible that lymph node metastasis could be predicted from the *HMBG1* gene expression levels, as determined from small-biopsy samples. However, the function of HMGB1 has not been clearly elucidated and further studies are needed to determine the mechanisms through which HMGB1 exerts its tumor-promoting effects in CRC.

In conclusion, we demonstrated that HMGB1 is a powerful prognostic marker in CRC and involved in mediating lymphatic invasion and lymph node metastasis. Our data suggest that investigation of HMGB1 expression in CRC tissues may help physicians to predict lymph node metastasis and clinical prognosis.

Conflicts of Interest

The Authors declare no conflicts of interest.

Acknowledgements

We thank K. Oda, M. Kasagi and S. Kono for their technical assistance. This work was supported in part by the following grants and foundations: CREST, Japan Science and Technology Agency (JST); and the Funding Program for Next Generation World-Leading Researchers (LS094).

References

1 Bustin M, Lehn DA and Landsman D: Structural features of the HMG chromosomal proteins and their genes. Biochim Biophys Acta *1049*: 231-243, 1990.

2 Scaffidi P, Misteli T and Bianchi ME: Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature *418*: 191-195, 2002.

3 Chen G, Li J, Ochani M, Rendon-Mitchell B, Qiang X, Susarla S, Ulloa L, Yang H, Fan S, Goyert SM, Wang P, Tracey KJ, Sama AE and Wang H: Bacterial endotoxin stimulates macrophages to release HMGB1 partly through CD14- and TNF-dependent mechanisms. J Leukoc Biol *76*: 994-1001, 2004.

4 Wang H, Vishnubhakat JM, Bloom O, Zhang M, Ombrellino M, Sama A and Tracey KJ: Proinflammatory cytokines (tumor necrosis factor and interleukin 1) stimulate release of high mobility group protein-1 by pituicytes. Surgery *126*: 389-392, 1999.

5 Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue KR, Faist E, Abraham E, Andersson J, Andersson U, Molina PE, Abumrad NN, Sama A and Tracey KJ: HMG-1 as a late mediator of endotoxin lethality in mice. Science *285*: 248-251, 1999.

6 Ostberg T, Kawane K, Nagata S, Yang H, Chavan S, Klevenvall L, Bianchi ME, Harris HE, Andersson U and Palmblad K: Protective targeting of high mobility group box chromosomal protein 1 in a spontaneous arthritis model. Arthritis Rheum *62*: 2963-2972, 2010.

7 Park S, Yoon SJ, Tae HJ and Shim CY: RAGE and cardiovascular disease. Front Biosci (Landmark Ed) *16*: 486-497, 2011.

8 Yang H and Tracey KJ: Targeting HMGB1 in inflammation. Biochim Biophys Acta *1799*: 149-156, 2010.

9 Evankovich J, Cho SW, Zhang R, Cardinal J, Dhupar R, Zhang L, Klune JR, Zlotnicki J, Billiar T and Tsung A: High mobility group box 1 release from hepatocytes during ischemia and reperfusion injury is mediated by decreased histone deacetylase activity. J Biol Chem *285*: 39888-39897, 2010.

10 Tang D, Kang R, Cao L, Zhang G, Yu Y, Xiao W, Wang H and Xiao X: A pilot study to detect high mobility group box 1 and heat shock protein 72 in cerebrospinal fluid of pediatric patients with meningitis. Crit Care Med *36*: 291-295, 2008.

11 Huang W, Tang Y and Li L: HMGB1, a potent proinflammatory cytokine in sepsis. Cytokine *51*: 119-126, 2010.

12 Campana L, Bosurgi L and Rovere-Querini P: HMGB1: a two-headed signal regulating tumor progression and immunity. Curr Opin Immunol *20*: 518-523, 2008.

13 Chen J, Xi B, Zhao Y, Yu Y, Zhang J and Wang C: High-mobility group protein B1 (HMGB1) is a novel biomarker for human ovarian cancer. Gynecol Oncol *126*: 109-117, 2012.

14 Ellerman JE, Brown CK, de Vera M, Zeh HJ, Billiar T, Rubartelli A and Lotze MT: Masquerader: high mobility group box-1 and cancer. Clin Cancer Res *13*: 2836-2848, 2007.

15 Kang R, Zhang Q, Zeh HJ, 3rd, Lotze MT and Tang D: HMGB1 in cancer: good, bad, or both? Clin Cancer Res *19*: 4046-4057, 2013.

16 Tang D, Kang R, Zeh HJ, 3rd and Lotze MT: High-mobility group box 1 and cancer. Biochim Biophys Acta *1799*: 131-140, 2010.

17 Washington MK: Colorectal carcinoma: selected issues in pathologic examination and staging and determination of prognostic factors. Arch Pathol Lab Med *132*: 1600-1607, 2008.
18 Volp K, Brezniceanu ML, Bosser S, Brabletz T, Kirchner T, Gottel D, Joos S and Zornig M: Increased expression of high mobility group box 1 (HMGB1) is associated with an elevated level of the antiapoptotic c-IAP2 protein in human colon carcinomas. Gut *55*: 234-242, 2006.
19 Xiang YY, Wang DY, Tanaka M, Suzuki M, Kiyokawa E, Igarashi H, Naito Y, Shen Q and Sugimura H: Expression of high-mobility group-1 mRNA in human gastrointestinal adenocarcinoma and corresponding non-cancerous mucosa. Int J Cancer *74*: 1-6, 1997.
20 Lange SS and Vasquez KM: HMGB1: the jack-of-all-trades protein is a master DNA repair mechanic. Mol Carcinog *48*: 571-580, 2009.
21 Travers AA: Priming the nucleosome: a role for HMGB proteins? EMBO Rep *4*: 131-136, 2003.
22 Lotze MT and Tracey KJ: High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nat Rev Immunol *5*: 331-342, 2005.
23 Taguchi A, Blood DC, del Toro G, Canet A, Lee DC, Qu W, Tanji N, Lu Y, Lalla E, Fu C, Hofmann MA, Kislinger T, Ingram M, Lu A, Tanaka H, Hori O, Ogawa S, Stern DM and Schmidt AM: Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases. Nature *405*: 354-360, 2000.
24 Tang D, Kang R, Coyne CB, Zeh HJ and Lotze MT: PAMPs and DAMPs: signal 0s that spur autophagy and immunity. Immunol Rev *249*: 158-175, 2012.

Received June 20, 2014
Revised July 22, 2014
Accepted July 23, 2014