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Clinical Implication of the Relationship Between High Mobility Group Box-1 and Tumor Differentiation in Hepatocellular Carcinoma

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Abstract. *Background/Aim: High mobility group box-1 (HMGB1) induces the release of proinflammatory cytokines and chemokines as a late-acting mediator of inflammation. Hepatocellular carcinoma (HCC) is a typical inflammation-related cancer. However, little is known about the relationship between HCC and HMGB1 and its receptor RAGE (receptor for advanced glycation end products). This study analyzes the clinicopathological relevance of HMGB1 expression level and the effect of HMGB1 expression on the characteristics of HCC. Materials and Methods: Samples from 75 HCC patients including 13 with positive hepatitis B surface antigen and 36 with hepatitis C antibody were studied. The expression of HMGB1 in paired cancer and non-cancerous tissues from patients with HCC was assessed using reverse-transcription polymerase chain reaction (RT-PCR) and western blotting. Quantitative RT-PCR data were analyzed in association with the clinicopathological factors of patients with HCC. Results: The expression of HMGB1 mRNA in HCC was high in well-differentiated tumors, but declined as tumors dedifferentiated to moderately and poorly differentiated HCC. The levels of HMGB1 mRNA showed a negative correlation with the presence of portal invasion (p=0.005) and the rise of serum PIVKA-II (p=0.034). There was no clear correlation between HMGB1 expression and proliferation activity of HCC using Ki-67 staining. Conclusion: In HCC, HMGB1 expression level correlated inversely with tumor differentiation. The RAGE-HMGB1 interaction may play a greater role in the early stages of HCC tumorigenesis than during cancer development.*

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Although many cancers arise from chronic inflammation, the relationships between carcinogenesis, cancer promotion, and its molecular characteristics remain poorly understood. Hepatocellular carcinoma (HCC), a typical inflammation-related tumor, is one of the most common malignancies in the world, especially in Asia and Africa. Japan has high incidences of chronic viral hepatitis, cirrhosis, and HCC. The details of inflammatory activity at the molecular level may be relevant in preventing hepatocarcinogenesis and promoting cancer.

High mobility group box-1 (HMGB1) protein was first recognized as one group of chromatin-associated proteins with high acidic and basic amino acid content and is composed of three domains (1). Its function as a cytokine-like factor is activated upon translocation from the cytoplasm into the nucleus where it binds DNA and regulates transcription (2). HMGB1 functions as a late mediator in various inflammatory processes (3). In its role as a late-acting mediator of inflammation, HMGB1 induces the release of proinflammatory cytokines and chemokines such as interleukin (IL)-12, IL-6, IL-1α, TNF-α, and IL-8 (4-6) by stimulating toll-like receptor ligands or LPS, TNF-α, and IL-1β (7) from macrophages (8, 9), dendritic cells (10), and natural killer cells (11). These cytokines cause diseases, including cancer (12-19), sepsis (9), arthritis (20), Alzheimer's disease (21), and ischemia-reperfusion injury (22).

Several receptors regarding HMGB1 have been identified, such as receptor for advanced glycation end products (RAGE), Toll-like receptor (TLR)2, and TLR4 (23) and thrombomodulin (24). Blocking the RAGE-HMGB1 interaction decreases tumor growth and metastasis, and suppresses the activation of p44/p42, p38, and SAPK/JNK MAP kinases (25), whereas it induces the phosphorylation of extracellular signal-regulated kinase (ERK) and nuclear translocation of nuclear factor (NF)-κB (26). TLR2 and TLR4 are also involved in HMGB1-induced NF-κB activation and neovascularization (22, 27-29). These molecular mechanisms are linked to cancer proliferation, invasion, and expression of matrix metalloproteinases.

The HMGB1-RAGE interaction has been demonstrated to promote various cancers, such as gastric cancer (12), colon cancer (13, 14), breast cancer (15), cutaneous malignancy (16, 17), and prostate cancer (18). We previously reported that the expression of *RAGE* mRNA, the representative receptor of HMGB1, was lower in normal liver than in hepatitis and higher than in HCC. The RAGE mRNA expression level was high in well-differentiated and moderately differentiated tumors but decreased in poorly differentiated HCC. *RAGE* mRNA was also upregulated in carcinogenesis but downregulated during HCC development (19). Moreover, hypoxia-resistant HCC cell lines had higher levels of RAGE expression, and significantly prolonged survival under hypoxia. Thus, the RAGE-HMGB1 interaction in HCC may have a function in the early stage of tumorigenesis and differ from the potential mechanism employed in other cancers. Ki-67 is a nuclear antigen present only in proliferating cells. A detailed cell-cycle analysis showed that the Ki-67 antigen is expressed in cells during the G_1, S, and G_1-M phases, but not during the G_0 phase of the cell cycle (30). This study examined cell proliferation activity of HCC and the relationship with HMGB1 expression by examining the expression of Ki-67. The clinicopathological relevance of the level of HMGB1 expression in patients with HCC was also examined.

Materials and Methods

Human samples. From October 2000 to December 2009, 110 patients with primary HCC were treated surgically in the Department of Surgical Oncology and Digestive Surgery, Kagoshima University. Of these 110 patients, 23 who had diabetes mellitus and 7 who underwent preoperative therapy were excluded from the study according to the results of our previous study (19) because RAGE expression is up-regulated in various tissues from patients with Diabetes (31, 32). Another five patients were excluded because their RNA samples were degraded. Samples from the remaining 75 patients (55 men and 20 women with a mean age of 68.2 years) were included in this study. Thirteen patients (17.3%) were positive for hepatitis B surface antigen and 36 (48.0%) were positive for the antibody to hepatitis C virus. Twenty-five patients (33.4%) were negative for both of these viruses and 1 patient (1.3%) was positive for both of these viruses. Thirty-three patients had chronic hepatitis and 31 had liver cirrhosis. The mean tumor size was 51.8 mm (range=13-150 mm). The histological grade of each tumor was determined according to the General Rules for the Clinical and Pathological Study of Primary Liver Cancer (The Liver Cancer Study Group of Japan, 2000). Fourteen tumors (18.6%) were well-differentiated HCC, 53 (70.7%) moderately differentiated HCC, and 8 (10.7%) poorly differentiated HCC.

Postoperative tumor recurrence was observed in 37 patients (49.3%). As a control study, 7 normal liver samples were collected from patients with benign or metastatic liver tumors. Before tissue acquisition, each patient provided written informed consent in a form recognized by the ethical committees of Kagoshima University School of Medicine.

Immunoblot analysis. Protein samples were prepared according to the Santa Cruz protocol. Lysates (30 μg) were subjected to immunoblot analysis using a 12.5% sodium dodecyl sulfate (SDS) -polyacrylamide gel followed by electrotransfer onto nitrocellulose filters. The filters were immunoreacted with an anti-HMGB1 antibody (SIGMA-ALDRICH, St. Louis, USA) and then incubated with peroxidase-conjugated anti-goat IgG (Medical and Biological Laboratories, Nagoya, Japan). The immune complex was visualized using an enhanced chemiluminescence western blot detection system (Pierce, Rockford, IL, USA). The amount of b-actin was also examined as an internal control using a specific antibody (Sigma-Aldrich, St. Louis, USA). At least three independent experiments were performed.

Quantitative RT-PCR. For reverse transcription-polymerase chain reaction (RT-PCR) and real-time quantitative PCR, total RNA was extracted from 30 mg frozen tissue using a Total RNA Mini Kit (VIOGENE, CA, USA). For cDNA synthesis, RNA samples (1 μg) were converted to cDNA by reverse transcription using random primers (TAKARA, Shiga, Japan) according to the manufacturer's instructions. To estimate the mRNA expression levels of several genes quantitatively, PCR amplification was performed using a Light-Cycler instrument system (Roche, Mannheim, Germany) and the Light-Cycler Fast Start DNA Master SYBR green I kit (Roche). Primers were as follows: *HMGB1* 5'-GCT CAG AGA GGT GGA AGA CCA-3' and 5'-GGT GCA TTG GGA TCC TTG AA-3' (21), *GAPDH* 5'-TTG GTA TCG TGG AAG GAC TCA-3' and 5'-TGT CAT ATT TGG CAG GTT T-3'. Amplification was carried out in 20 μl reactions containing 4 mM $MgCl_2$, 2 μl of primers, 2 μl of Light-Cycler Fast Start DNA Master SYBR green I reagent, and 2 μl of cDNA. Reaction conditions were as follows: initial incubation at 95˚C for 10 min followed by 50 cycles at 95˚C for 10 sec for denaturation, 54˚C for 10 sec for annealing of HMGB1 primers or 60˚C for 10 sec for annealing of GAPDH primers, and 72˚C for 10 sec for extension. Melting curves were obtained according to the protocol under the following conditions: 0 sec denaturation period at 95˚C, starting temperature of 65˚C, final temperature of 95˚C, and rate of temperature increase of 0.1˚C per sec. The quantitative value of the target gene in each sample was normalized using GAPDH expression as an internal control. The quantitative RT-PCR assay was carried out twice and the mean value was calculated. Finally, the mRNA expression ratio of cancerous (C) to non-cancerous (N) tissue was calculated using the following formulae: R=log {target gene (C)/GAPDH (C)}, R=log {target gene (N)/GAPDH (N)}. These experiments were carried out twice to confirm reproducibility.

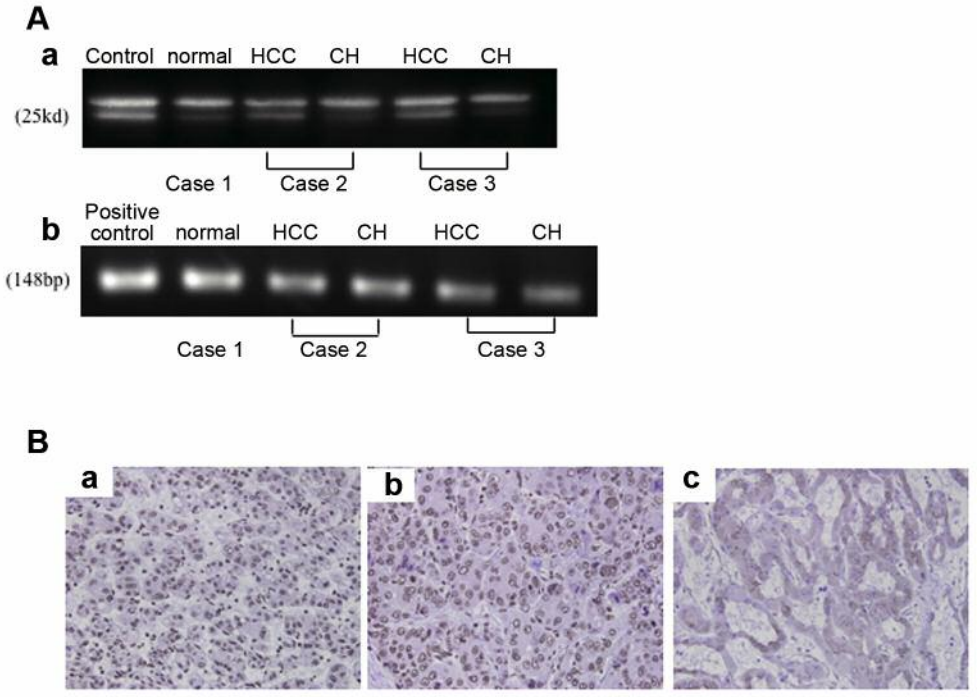

Figure 1. *HMGB1 expression. (A) HMGB1 expression by western blotting (a) and RT-PCR (b). The expression in normal liver (case 1) and paired cancer and noncancerous tissues (case 2 and 3) was represented. Normal: Normal liver; CH: chronic hepatitis. (B) HMGB1 expression by immunohistochemical staining: (a) well differentiated HCC, (b) moderately differentiated HCC, (c) poorly differentiated HCC.*

Immunohistochemistry. Consecutive 4-μm sections were cut from each paraffin-embedding block. Sections were immunostained by an anti-HMGB1 antibody (SIGMA, CA, USA) and anti-Ki-67 (MIB-1, Dako, Glostrup, Denmark) according to the conventional immunoperoxidase technique. After peroxidase-blocking with 3% H_2O_2/methanol for 10 min and autoclave treatment (121˚C, 10 min), specimens were blocked with phosphate-buffered saline (PBS) containing 5% normal horse serum (Vector Laboratories, Inc., Burlingame, CA, USA). An anti-HMGB1 antibody was used at 1:200 and anti-Ki-67 was used at 1:100. After overnight incubation at 4˚C with the primary antibody, specimens were briefly washed in PBS and incubated at room temperature with peroxidase-conjugated secondary antibody. The specimens were then washed in PBS and color-developed by diaminobenzidine solution (DAKO). After washing with water, specimens were counterstained with Meyer's hematoxylin (Sigma Chemical Co., St. Louis, MO, USA). Immunostaining of all cases was performed at one time to ensure uniform conditions of antibody reaction and DAB exposure. To evaluate the immunohistochemical staining, ten fields were selected and expression in all tumor cells was evaluated with high-power (×200) microscopy.

Statistical analysis. Statistical analysis was performed using the JMP IN version 5.1.2 software system (SAS Institute Inc., NC, USA). Gene expression was compared among normal liver, hepatitis, and HCC using Student's *t*-test. The relationships between *HMGB1* mRNA expression levels and clinicopathological factors were evaluated using the Student's *t*-test and the Mann-Whitney *U*-test, as appropriate. A *p*-value of less than 0.05 was considered statistically significant.

Results

HMGB1 expression in HCC. Protein and mRNA expression of HMGB1 were examined by western blotting and RT-PCR in normal liver, noncancerous, and cancerous tissues from three cases (Figure 1A). All three cases showed expression of HMGB1 protein and mRNA in these tissues. The expression of HMGB1 in tumor cells was examined by immunohistochemical staining. HMGB1 expression was observed in the nucleus (Figure 1B).

Quantitative HMGB1 mRNA expression in HCC and noncancerous lesions. Quantitative expression of *HMGB1* mRNA was compared in paired cancer and noncancerous tissues (*i.e.*, tissues from patients with chronic hepatitis or liver cirrhosis) from 75 cases, and from seven normal liver cases. The mean value in cancerous tissues and noncancerous tissues tended to be higher than that in normal liver tissues, although the differences were not significant (Figure 2).

Relationship between HMGB1 mRNA expression and clinicopathological factors. To elucidate the biological significance of HMGB1 expression in HCC, the levels of *HMGB1* mRNA expression were compared with the

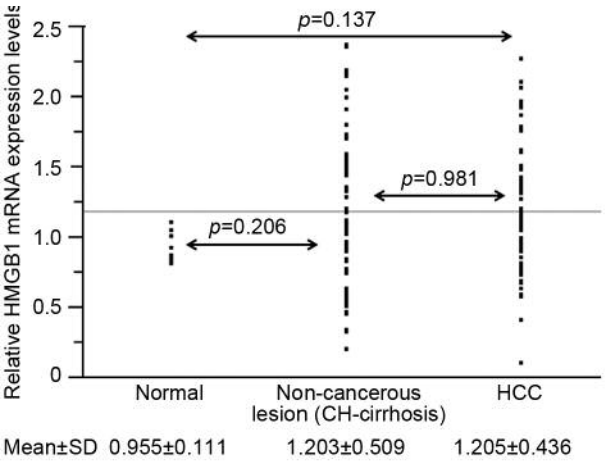

Figure 2. *Quantitative expression of HMGB1 in normal liver (n=7) and paired noncancerous and cancerous tissues (n=75).*

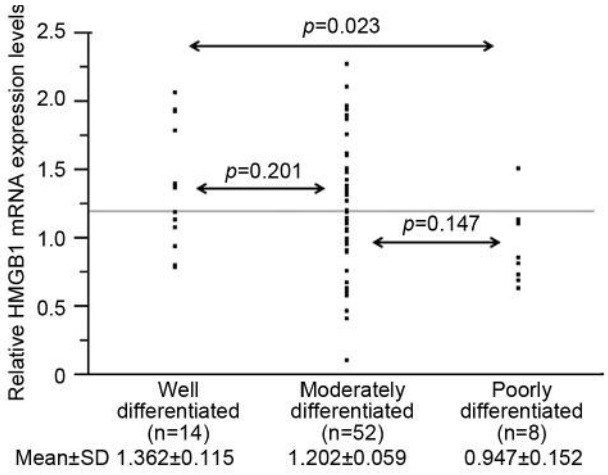

Figure 3. *Quantitative expression of HMGB1 according to tumor differentiation.*

clinicopathological factors of 75 patients. As shown in Table I, significant differences were noted in *HMGB1* mRNA expression in association with portal invasion, tumor differentiation, and elevated levels of protein induced by Vitamin K absence or its antagonist II (PIVKA-II). In the patients with portal vein invasion, *HMGB1* expression was lower than levels in patients without portal vein invasion. *HMGB1* expression was lower in patients with poorly differentiated HCC than in patients with well-differentiated HCC.

Relationship between HMGB1 expression and proliferation activity of HCC according to tumor differentiation. When comparing HMGB1 expression according to tumor differentiation, the level of HMGB1 expression decreased as

Table I. *Relationship between tumor HMGB1 expression and clinicopathologic features.*

Factors	HMGB1 mRNA expression				*p*-Value
	n	mean±SD			
Gender					
Male	55	1.195±0.459			0.762
Female	20	1.230±0.379			
Age					
<65 years	19	1.236±0.481			0.717
≥65 years	56	1.194±0.425			
Viral hepatitis status					
HBsAg, positive	13	1.344±0.544	0.211		0.356
Anti-HCV-Ab, positive	36	1.164±0.398	0.211	0.802	0.356
Non-viral	25	1.190±0.443		0.802	0.356
Tumor size(mm)					
<30	21	1.165±0.405			0.627
≥30	54	1.220±0.451			
Growth form					
Expansive	72	1.212±0.435			0.473
Invasive	3	1.026±0.528			
Capsule formation					
Absent	8	1.387±0.310			0.215
Present	67	1.183±0.446			
Capsule invasion					
Absent	15	1.362±0.387			0.119
Present	60	1.165±0.443			
Septum formation					
Absent	11	1.379±0.455			0.152
Present	64	1.175±0.430			
Vascular invasion					
Absent	41	1.280±0.472			0.099
Present	34	1.113±0.377			
Portal invasion					
Absent	44	1.322±0.432			0.005
Present	31	1.037±0.392			
Venous invasion					
Absent	57	1.235±0.444			0.284
Present	18	1.108±0.408			
Bile duct invasion					
Absent	70	1.221±0.423			0.233
Present	5	0.977±0.608			
Tumor differentiation					
Well	14	1.362±0.115	0.23		0.025
Moderately	53	1.202±0.059	0.23	0.122	0.025
Poorly	8	0.947±0.152		0.122	0.025
TMN stage*					
I-II	39	1.298±0.453			0.051
III-IV	36	1.103±0.400			
PIVKA-II					
Normal	22	1.375±0.399			0.034
High	51	1.137±0.444			
AFP					
Normal	29	1.296±0.462			0.149
High	46	1.147±0.415			
Recurrence					
Yes	37	1.163±0.416			0.522
No	38	1.232±0.464			

*According to the criteria of the Liver Cancer study Group of Japan. HBsAg: Hepatitis B surface antigen; HCV: hepatitis C virus; Ab: antibody; PIVKA-II: protein induced by Vitamin K absence or antagonists-II; AFP: alpha-fetoprotein.

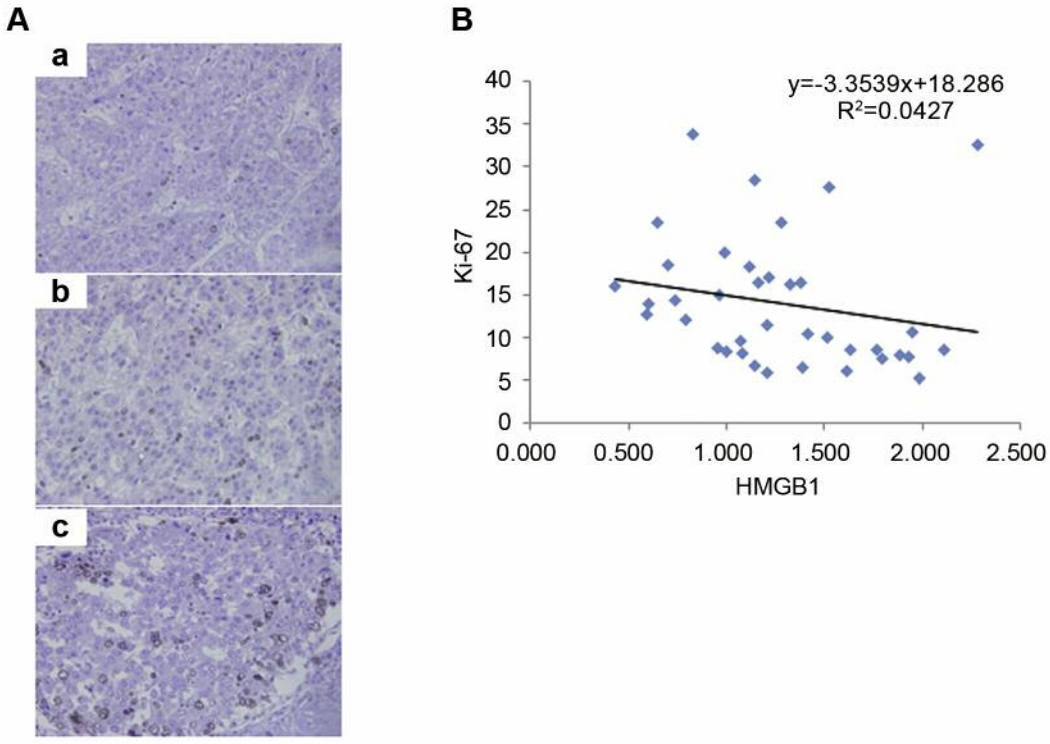

Figure 4. *Ki-67 expression and relationship between HMGB1 and Ki-67. (A) Ki-67 expression by immunohistochemical staining: (a) well differentiated HCC, (b) moderately differentiated HCC, (c) poorly differentiated HCC. (B) Correlation of HMGB1 expression and proliferation activity of HCC by Ki-67 staining.*

HCC became less differentiated (Figure 3). Moreover, the proliferation activity of HCC was examined by immunohistochemical Ki-67 staining (Figure 4A). The rate of Ki-67 positive cells (namely, proliferation activity of HCC) increased as HCC became less differentiated. There was no clear correlation between HMGB1 expression and proliferation activity of HCC (Figure 4B).

Relationship between HMGB1 expression in HCC and patient survival. In order to show the relationship between HMGB1 expression and patient prognosis, we divided the 75 patients were divided into 2 groups by the median level of HMGB1 mRNA expression. When comparing overall and recurrence-free survival, there were no significant differences in HMGB1 expression (Figure 5).

Discussion

When inflammatory events such as injury, infection, or cell necrosis occur, HMGB1 is secreted (33, 34). It induces the release of pro-inflammatory cytokines and chemokines such as p44/p42, p38, SAPK/JNK MAP kinases, ERK, and NF-κB (25). Therefore, we hypothesized that HMGB1 may play an important role in the occurrence and development of HCC, which typifies inflammation-related tumors caused by viral or alcoholic hepatitis. Results from our previous study showed the clinicopathological relevance of the *RAGE* mRNA expression level, the counter-receptor for *HMGB1*, in patients with HCC (19). In that study, significant differences in *RAGE* mRNA expression were shown according to gender, age, the level of PIVKA-II, tumor differentiation, and postoperative recurrence of HCC. In particular, the level of RAGE mRNA expression was higher in well or moderately differentiated tumors than in poorly differentiated tumors, and this result was confirmed by immunohistochemical examination. Moreover, the levels of *RAGE* mRNA showed an inverse correlation with the presence of recurrence.

In the present study, comparing the quantitative expression of *HMGB1* mRNA in paired cancerous and non-cancerous tissues (*i.e.* chronic hepatitis or liver cirrhosis), there was no significant difference between cancerous tissues and non-cancerous tissues. The mean values of *HMGB1* mRNA expression in cancerous and non-cancerous tissues tended to be higher than that in normal liver tissues (*i.e.* non-inflamed liver from benign or metastatic liver tumor patients). Furthermore, in patients with HCC, the *HMGB1* mRNA level differed significantly with regard to tumor differentiation. *HMGB1* mRNA was expressed at lower level in poorly

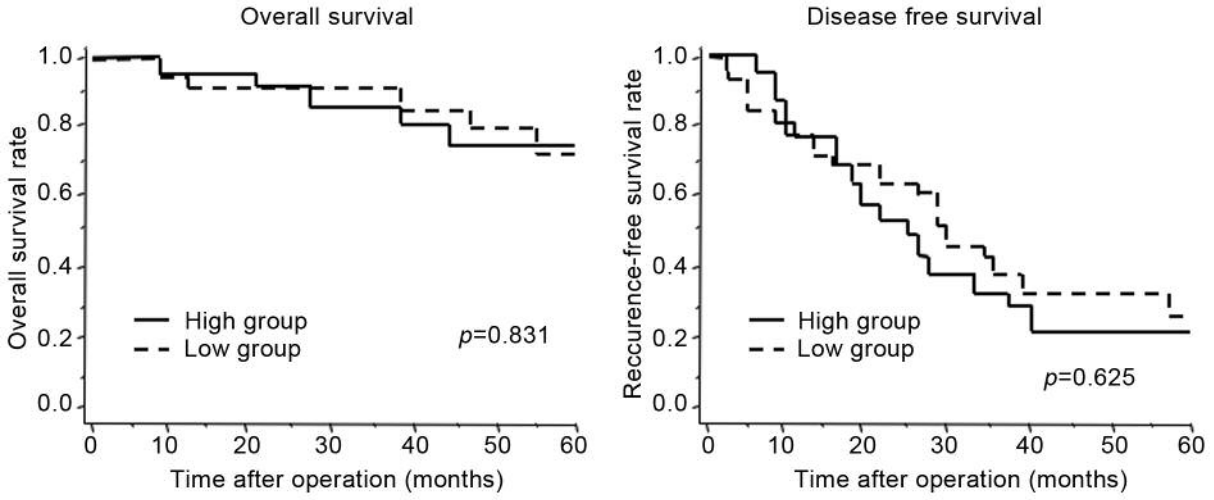

Figure 5. *Relationship between HMGB1 expression in HCC and patient's survival. All patients were divided into the 2 groups by the median level of HMGB1 mRNA expression.*

differentiated HCC than in well differentiated HCC (p=0.023). The levels of *HMGB1* mRNA showed a negative correlation with the presence of the portal invasion (p=0.005) and the rise of serum PIVKA-II (p=0.034). These results were remarkably similar to those of our previous study of the clinicopathological relevance of the level of RAGE expression (19). Whereas HMGB1 has been demonstrated to promote various cancers, Akaike *et al*. evaluated expression of HMGB1 in gastric cancer and reported that the prognosis of the low HMGB1 group was significantly poorer than that of the high HMGB1 group and HMGB1 expression revel in gastric cancer tended to be correlate inversely with depth of tumor invasion and lymph node metastasis (35). Cebrián *et al*. also reported in review of the biological and clinical relevance of HMGB1 in pancreatic cancer that HMGB1 exhibits a dual and paradoxical role; that is, extracellular HMGB1 becomes a pro-tumor protein, acting as cytokine, chemokine and growth factor, whereas intracellular HMGB1 functions as an anti-tumor protein, stabilizing the genome and sustaining autophagy (36).

Once cancer is established, HCC differentiates systematically to a more malignant histology, from well or moderately differentiated to poorly differentiated HCC. In our results, when HCC is established (and is well differentiated), HMGB1 and RAGE expression are high. This may reflect elevated tumor activity as shown in Figure 6. Evidence from clinical studies (37-39) indicate that well-differentiated HCC consists of hypovascular tumors primarily fed by the portal vein system, while moderately and poorly differentiated HCC consist of hypervascular tumors primarily fed by arterial blood. Considering the level of *RAGE* mRNA

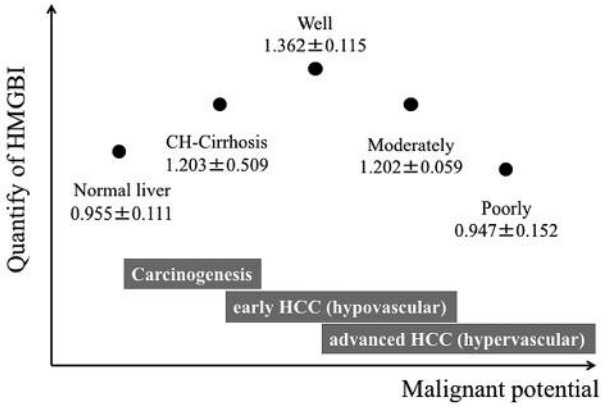

Figure 6. *Scheme of the change of HMGB1 expression according to the sequential change of liver tissue: normal → chronic hepatitis (CH) → cirrhosis → HCC. The value indicates the quantitative HMGB1 mRNA expression.*

expression in each stage of differentiation and the *in vitro* results, it is suggested that RAGE has a function in resistance to hypoxia. HMGB1 might be also be related to hypoxia resistance leading to the induction of angiogenic cytokines such as IL-8. The HMGB1-RAGE interaction undoubtedly plays a vital role in HCC.

In conclusion, HMGB1 expression level in HCC correlated inversely with tumor differentiation and was similar to the level that we reported previously in a study on the clinicopathological relevance of RAGE level in HCC. The RAGE-HMGB1 interaction might work in the early stage of HCC tumorigenesis than in the stage of cancer

development. The interaction of RAGE and HMGB1 in cancer is still poorly understood, and it is expected that further examination of their molecular mechanisms will aid in the development of new cancer therapies.

Conflicts of Interest

The Authors declare no potential conflicts of interest.

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