ORIGINAL ARTICLE

High Mobility Group Box 1 (HMGB1) expression in gastric adenocarcinomas

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Summary

Purpose: High mobility group box 1 (HMGB1), the most important member of the high mobility group box protein family, is a nuclear protein with different functions in the cell; it has a role in cancer progression, angiogenesis, invasion, and metastasis development. The purpose of this study was to investigate the expression of HMGB-1 in gastric adenocarcinoma (GC) and to evaluate its diagnostic and prognostic value.*

Methods: This study included 85 cases histopathologically diagnosed with GC. Sections obtained from formalin-fixed paraffin-embedded blocks were stained immunohistochemically with HMGB1 antibody. HMGB1 expression was compared with clinicopathologic and prognostic data.*

Results: HMGB1 expression was negative in 16 (18.8%) patients and positive in 69 (81.2%) patients. There was no correlation between HMGB1 expression and age, sex, histologic subtype of tumor, lymph node involvement (p=0.455, p=0.365, p=0.448, p=0.077, respectively). There was a significant correlation between HMGB1 expression and tumor grade, local invasion depth (T stage) and pTNM stage (p=0.016, p=0.022, p=0.015, respectively).*

Conclusion: It was found that in the presence of HMGB1 expression, the grade of tumor differentiation decreased and the depth of invasion increased, which was associated with higher stage. These findings suggest that HMGB1 is an independent prognostic factor for GC.*

Key words: gastric adenocarcinoma, HMGB1, immunohistochemistry, prognosis

Introduction

Despite the emerging data that indicate a declining incidence and mortality, GC is still one of the most common malignancies in the world causing around 738,000 deaths worldwide [1]. Unfortunately the majority of these cancers at the time of diagnosis are at advanced stage and the treatment options are limited. A better understanding of the mechanism contributing to GC initiation and progression is searched with the hope to improve early diagnosis and treatment efficacy. Thus, the need for identification of prognostic and early detection biomarkers possessing predictive value for survival of GC patients, is of paramount importance.

HMGB proteins are non-histone nuclear proteins with many different functions in the cell. HMGB1, HMGB2, and HMGB3 are the members of the HMGB protein family. While the expressions of HMGB2 and HMGB3 are limited, HMGB1 expression is common [2]. HMGB1 binds DNA's small groove nonspecifically and modifies the interaction of some transcription factors such as p53 and steroid hormone receptors with DNA. It plays a role in DNA repair, transcription, differentiation, extracellular signaling and somatic recombination [3]. It also functions as an extracellular signaling molecule releasing from necrotic and inflamma-

tory cells. Extracellularly bounding receptors are RAGE (receptor for advanced glycation end product), TLR-2 (Toll-like receptor-2), TLR-4 and TLR-9 [4]. RAGE acts as a signaling molecule in inflammation, cell differentiation, cell migration and tumor migration mediating the intracellular effects of extracellular HMGB1 [5,6].

HMGB1 overexpression has been shown in several tumor types such as skin squamous cell carcinoma, prostate carcinoma, colorectal carcinoma, breast carcinoma, bladder carcinoma and ovarian carcinoma [7-14].

Our aim was to investigate the presence of HMGB1 expression in GC cases and to see for any correlation with clinicopathological and prognostic data.

Methods

Selection of patients

This study included 85 cases that were histopathologically diagnosed with GC upon subtotal or total gastrectomy material and followed up at Health Sciences University, Antalya Education and Research Hospital between January 2010 and July 2014. The study was approved by the local ethics committee. All hematoxylin & eosin (H&E) stained samples were re-evaluated by the authors (DS,AAG) according to AJCC 7th edition. The age and gender of the patients, type of surgery were obtained from patient files.

Tissue preparation and immunohistochemical staining

Tissue samples obtained just after gastrectomy were immediately fixed in 10% formaldehyde and embedded in paraffin. Then, 4 μm thick sections were obtained from paraffin blocks and were stained with H&E for initial assessment. Cross sections of 4μm thickness prepared for immunohistochemical staining were deparaffinized in oven at 60°C for 2 hrs. Afterwards, they were kept in xylene for 30 min, and in gradient ethanol for 30 min (70% ethanol for 10 min, 96% ethanol for 10 min, 100% ethanol for 10 min) and washed with tap water. Next, the tissue sections were heated in a 10% citrate buffer solution (#RE7113; Leica Microsystems, Inc., Milton Keynes, UK) in the microwave at 800 W for 10 min and then at 400 W for an additional 10 min. Sections were brought out of the microwave and allowed to cool at room temperature for 30 min. Endogenous peroxidase activity was neutralized by incubation in 3% hydrogen peroxide for 10 min. Sections were washed with phosphate buffered saline (PBS) for 2 min. Then, sections were incubated with primary antibodies against HMGB-1 (#EPR3506; dilution 1:100; Abcam, Lab Vision, Cambridge, MA, USA) for 60 min. Afterwards, they were treated with secondary antibody (Biotinylated Goat antirabbit Immunoglobulin secondary antibody; #BP9100; ready to use; Vector Laboratories, Burlingame, CA, USA) for 20 min at 30°C and washed with PBS for 5 min. Sections were then incubated with conjugated peroxidase (#RE7110K; Novocastra; Leica Microsystems Inc., USA) for 20 min and then washed with PBS for 5 min and were kept in chromogenic 3,3'diaminobenzidine for 5 min. Sections were washed under tap water and counterstained with hematoxylin. Then, the tissue samples were dehydrated, dried and covered with Entellan®.

Microscopic examination of hematoxylin & eosin-stained sections

In all cases, tumor type, tumor subtype, tumor grade, level of tumor depth and lymph node metastasis status were assessed.

Evaluation of immunohistochemically stained sections

Expression rates for the positive tumor cells in the specimens were evaluated by 2 pathologists (DS,AAG) who were unaware of the patients' clinical features. Although there was no HMBG1 expression on non-neoplastic gastric surface epithelium and gland epithelium, there was a strong nuclear staining in lymphoid follicles in the stroma. This nuclear staining observed in lymphocytes was used as the positive internal control in the evaluation of cases. Vascular structures, fibroblasts, smooth-muscle cells, vessel endothelium, vessel wall, neural structures, and adipocytes within the crosssection showed no staining. Absence of expression in these
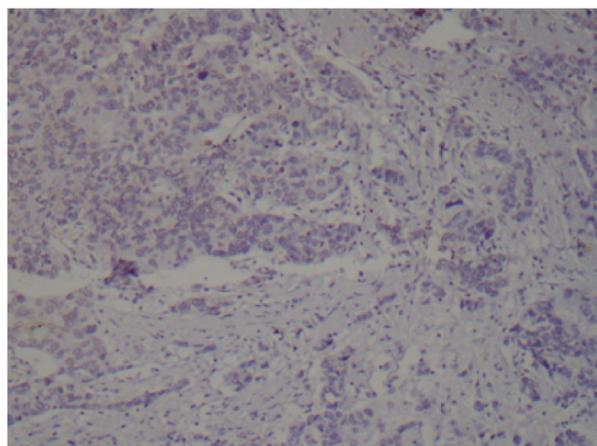
Figure 1. Negative expression of HMGB1 in gastric adenocarcinoma (score 0) (HMGB1 ×100).
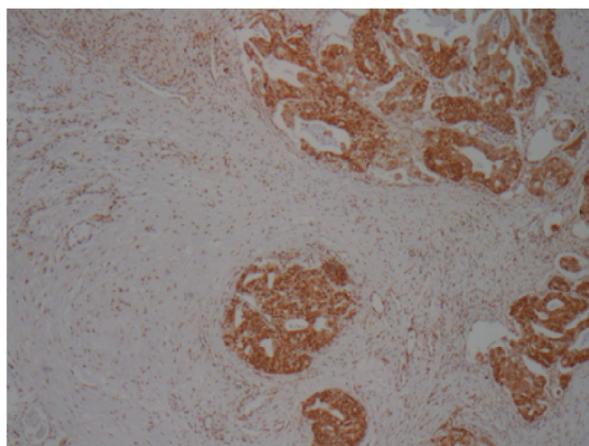
Figure 2. Diffuse HMGB-1 positive expression in gastric adenocarcinoma (score 3) (HMGB1 ×40).

structures was used as the negative internal control in immunohistochemical evaluation. In carcinoma cases with HMGB1 expression, staining was nuclear and accompanied by weak cytoplasmic staining. This cytoplasmic staining was ignored and nuclear staining was evaluated. Nuclear staining in <5% of tumor cells rated as Score 0, nuclear staining in 5-24% of tumor cells rated as Score 1, nuclear staining 25-49% of tumor cells rated as Score 2 and nuclear staining 50-100% of tumor cells rated as Score 3. For statistical analysis, Score 0 was evaluated as negative expression and scores 1-2-3 were evaluated as positive expression (Figures 1,2).

Statistics

Statistical analyses were performed using SPSS for Windows 15.0 software. Distribution of the variables normality was examined using visual (histogram and probability plots) and analytical methods (Kolmogorov-Smirnov/Shapiro-Wilk tests). Normal distribution was accepted when p value was greater than 0.05 in the Kolmogorov-Smirnov test. Differences between groups were examined using Chi-square and Mann-Whitney test. Statistical significance was set at $p < 0.05$.

Results

Clinicopathological characteristics

A total of 85 patients, 34 (40%) of whom were female and 51 (60%) male were included in the study. The mean age of the patients was 65.5 ± 12.9 years (range 33-91). Histopathological evaluation of the patients revealed tubular adenocarcinoma in 72 (84.7%) patients and signet ring cell carcinoma in 13 (15.3%). When tumor differentiation was evaluated, 10 (11.8%) patients had well differentiated, 26 (30.6%) had moderately differentiated, and 49 (57.6%) patients had poorly differentiated tumors. T1 disease was detected in 14 (16.5%) patients, T2 in 9 (10.6%), T3 in 46 (54.1%) and T4 in 16 (18.8%) patients. N0 disease was detected in 30 (35.3%) patients, N1 in 17 (20%), N2 in 12 (14.1%) and N3 in 26 (30.6%) patients. Seventeen (20%) patients had stage 1, 31 (36.5%) had stage 2 and 37 (43.5%) patients had stage 3 when evaluated according to the final stages.

Table 1. Relationship between HMGB-1 expression and tumor differentiation

HMGB1 expression	Grade of differentiation			Total
	Grade 1 n (%)	Grade 2 n (%)	Grade 3 n (%)	
HMGB1 Negative	5 (50)	6 (23.1)	5 (10.2)	16 (18.8)
HMGB1 Positive	5 (50)	20 (76.9)	44 (89.8)	69 (81.2)
Total	10 (100)	26 (100)	49 (100)	85 (100)

Table 2. Relationship between HMGB-1 expression and T stage

HMGB1 expression	T Stage				Total
	T1 n (%)	T2 n (%)	T3 n (%)	T4 n (%)	
HMGB1 Negative	6 (42.9)	3 (33.3)	4 (8.7)	3 (18.8)	16 (18.8)
HMGB1 Positive	8 (57.1)	6 (66.7)	42 (91.3)	13 (81.3)	69 (81.2)
Total	14 (100)	9 (100)	46 (100)	16 (100)	85 (100)

Table 3. Relationship between HMGB-1 expressıon and pTNM stage

HMGB1 expression	pTNM stage			Total
	Stage 1 n (%)	Stage 2 n (%)	Stage 3 n (%)	
HMGB1 Negative	7 (43.8)	6 (37.5)	3 (18.8)	16 (100)
HMGB1 Positive	10 (14.5)	25 (36.2)	34 (49.3)	69 (100)
Total	17 (20)	31 (36.5)	37 (43.5)	85 (100)

Immunohistochemical study findings

Immunohistochemical HMGB1 expression was negative in 16 (18.8%) patients and positive in 69 (81.2%) patients. There was no statistically significant relationship between HMGB1 expression and age, sex, histological subtype of the tumor, lymph node involvement (p=0.455, p=0.365, p=0.448 and p=0.077, respectively). There was a significant negative correlation between tumor differentiation and HMGB1 expression (p=0.016; Table 1). A significant relationship was noticed between HMGB1 expression and T stage (p=0.022; Table 2) and also a significant relationship between HMGB1 expression and TNM stage (p=0.015; Table 3).

Discussion

In this study our aim was to investigate the diagnostic and prognostic significance of immunohistochemical expression of HMGB1 in GC cases and therefore the relationship between age, sex, histologic tumor subtype, tumor grade, tumor invasion depth (T stage), lymph node involvement (N stage), TNM stage and immunohistochemical expression of HMGB1 were investigated.

HMGB1 also enhances the activity of some transcription factors related with cancer development. These include p53, p73, retinoblastoma protein, transcription factors such as Rel/NFkB family, and estrogen receptor, which is a nuclear hormone receptor [15-17]. HMGB1 has paradoxical effects in carcinogenesis. It stimulates tumor neo-angiogenesis and enhances protective anti-tumor T-cell response [18]. HMGB1 released from dead tumor cells stimulates mature dendritic cells and completes the tumor antigen presentation process by interacting with TLR-4, so it enhances the immune response against the tumor [19].

HMGB1 is able to inhibit apoptosis by different pathways. HMGB1 overexpression suppresses caspase-3 and caspase-9 activity, inhibiting thus significant steps in apoptosis. HMGB1 overexpression was shown to regulate c-IAP2, which is an antiapoptotic protein. In colorectal cancer, cytochrome apoptosis inhibitor protein 2 (c-IAP2) levels are related to HMGB1 expression [20]. Cell line studies indicate that HMGB1 inhibits the expression of Bak, which is a member of the proapoptotic Bcl-2 family [11].

HMGB1 is associated with the pathological stage of a tumor. Real-time PCR showed an increase in HMGB1 mRNA expression as tumor stage rises. Because of these reasons, HMGB1 and its receptor, RAGE, have become important in target treatment. Blockage of RAGE, which mediates the extracellular effects of HMGB1, may inhibit growth or progression of tumors. Various strategies have been evaluated for blocking the HMGB1 signal, such as management of the extracellular ligand-binding section of sRAGE, blockage of Fab fragments derived from anti-RAGE, and/or anti-HMGB1 IgG [21].

Evidence supporting the role of HMGB1 in cancer progression, angiogenesis, invasion, and metastasis development has been steadily accumulating [22,23]. The relation of HMGB1 overexpression with presence of lymph node metastasis and advanced stage in colorectal carcinoma, ovarian carcinoma, head-neck carcinoma and esophageal squamous cell carcinoma was demonstrated in many studies [9,14,22,23]. In our study, there was a significant relation determined between HMGB1 and tumor grade, level of tumor infiltration depth (T stage) and TNM stage.

HMGB1 expression studies in GC are rare. Bao et al. found overexpression of HMGB1 in cancer tissues with strong reactivity rate, compared with noncancer tissues. No significant correlation was found between HMGB1 overexpression with age, gender or TNM staging when the cases were divided into two groups: early stage (I + II) and late stage (III +IV). In our study, we found that HMGB 1 expression was significantly correlated with T stage and final stage. Statistically significant difference was found in the groups with different differentiation similar to our study. In our study there was a significant negative correlation between tumor differentiation and HMGB1 expression (p=0.016). Bao et al. found significant difference in disease-free survival between groups with HMGB1 overexpression and low-level expression [24]. The expected disease-free survival was 20.4375±7.28648 months for tumors with HMGB1 overexpression (95% CI=17.8104-23.0646), and 15.5870±8.23158 months for tumors without HMGB1 expression or low-level expression (95% CI=13.1425-18.0314).

Chung et al. found that serum HMGB1 levels were significantly associated with the depth of invasion, lymph node metastasis, tumor size, and poor prognosis in their study that investigated the the correlation between the serum HMGB1 levels and the clinical and pathologic features of GC [25]. While this study investigated the serum levels of HMGB1, lymph node metastasis was significantly associated with serum HMGB1 levels, different from our study.

Akaike et al. investigated the immunohistochemical expression of HMGB-1 in GC cells and the relation with clinicopathological findings in 76 cases with primary GC. Each patient was classified as high HMGB-1 or low HMGB-1 according

to HMGB-1 expression (above or below the mean of 74.6 per 200 cancer cells, respectively) in their study. The authors reported that the expression of HMGB-1 in GC cells with the intestinal type was significantly increased compared to the diffuse type [26]. In our study, we didn't find such a difference. In their study, unlike Chung et al. and our study, the prognosis of the low HMGB-1 group was significantly poorer compared with the high HMGB-1 group. The difference between antibody clone, evaluation of staining or patient groups may be the reason for the difference with our study.

Conclusion

In GC cases, we found that in the presence of HMGB1 expression, the grade of tumor differentiation decreased and the depth of invasion increased, which was associated with higher stage. These findings suggest that HMGB1 is an independent prognostic factor for GC.

Conflict of interests

The authors declare no conflict of interests.

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