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Original Article

Expression of HMGB1/RAGE protein in renal carcinoma and its clinical significance

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Abstract: Objective: To investigate the expression of high mobility group protein B1 (HMGB1) and its receptor, receptor for advanced glycation end-product (RAGE), in renal cancer tissue and surrounding normal tissue and to analyze the relationship between the expression level of the protein and receptor as well as the clinical pathological characteristics and prognosis in renal cancer patients. Methods: A total of 80 renal carcinoma patients who were surgically treated in our hospital from February 2004 to December 2012 were included in this study. Normal paratumoral tissues were collected as a control. All diagnoses were confirmed with a postoperative pathological examination. All patients had complete pathological data. The expression of HMGB1/RAGE proteins in renal cancer tissue and paratumoral tissue was examined using immunohistochemical methods. Results: The positive expression rate of HMGB1 was 71% in renal cancer tissue, which was significantly higher than that in the paratumoral normal tissue (25%). The positive expression rate of RAGE was 72% in renal cancer tissue, which was significantly higher than that in the paratumoral normal tissue (27%). Further analysis did not indicate a correlation between the positive expression of HMGB1 and RAGE proteins and gender, age and tumor size ($P > 0.05$), whereas the expression patterns were shown to correlate with tumor differentiation, clinical stage and lymph node metastasis ($P < 0.05$). The expression of HMGB1 exhibited a significant positive correlation with RAGE level ($P < 0.05$), the expression of HMGB1/RAGE proteins exhibited a negative correlation with the prognosis of patients, and the five-year survival rate of patients with positive expression was significantly lower than that of patients with negative expression ($P < 0.05$). Conclusion: HMGB1/RAGE exhibited significantly elevated expression in renal cancer tissues that was closely related to the clinical prognosis of patients; thus, the expression levels may become a new target in the treatment of renal carcinoma.

Keywords: Renal cell carcinoma, HMGB1, RAGE

Introduction

Renal carcinoma is the most common malignant renal tumor, and it has variable biological characteristics and is prone to metastasis and relapse [1]. In recent years, the incidence of renal carcinoma shows a rising trend over each year. Because of highly expressed drug-resistant genes in renal carcinoma cells, routine chemotherapy has no significant effect in renal carcinoma. Therefore, developing a deeper understanding of the mechanisms underlying renal carcinoma pathogenesis and progression along with specifically targeted drugs has important clinical significance in the improvement of prognoses in renal carcinoma patients. Previous studies have found that high mobility group protein B1 (HMGB1) and its receptor, receptor for advanced glycation end-product (RAGE), play important roles in the development, progression and metastasis of multiple tumors [2, 3]. This study intended to provide a new direction for the treatment of renal carcinoma through analyses of the expression of HMGB1 and RAGE proteins in renal cancer tissue and the clinical significance of such expression.

Materials and methods

General information

A total of 80 renal carcinoma patients who were surgically treated in our hospital from February 2004 to December 2012 were included in this

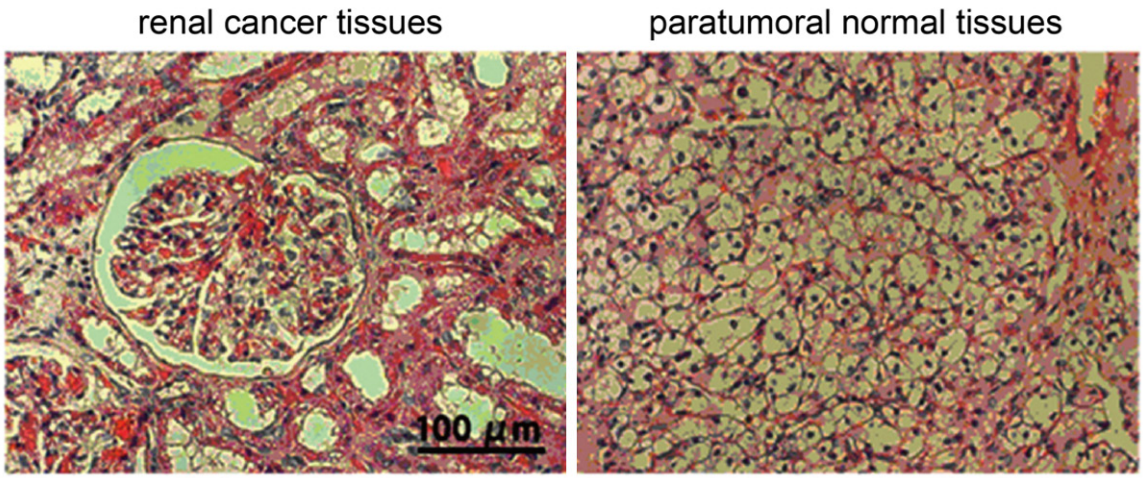

Figure 1. HE staining of renal cancer tissues and paratumoral normal tissues.

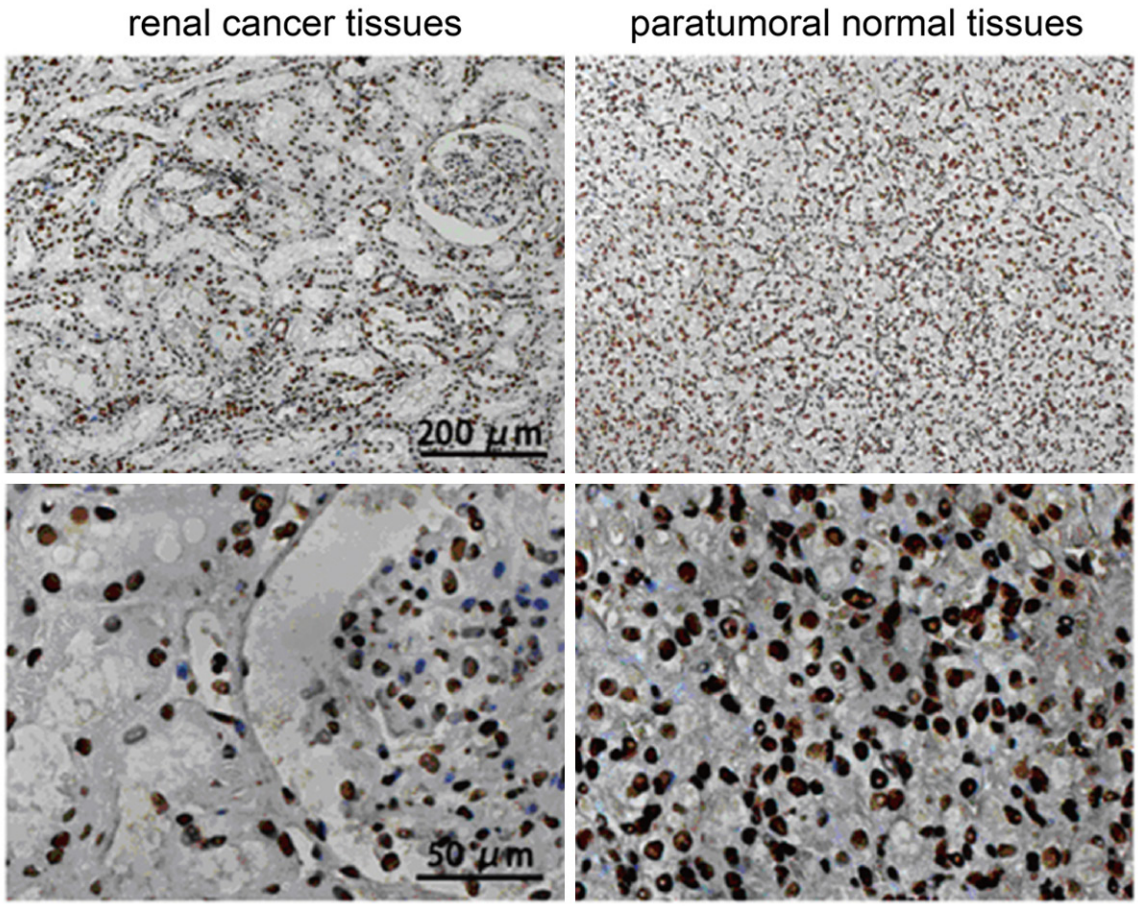

Figure 2. Typical immunohistochemistry staining results of HMGB1 expression in renal cancer tissues and paratumoral normal tissues.

study. Immunohistochemistry was performed with surgically resected tumor tissues and adjacent normal tissues. Diagnoses were confirmed with postoperative pathological exami-

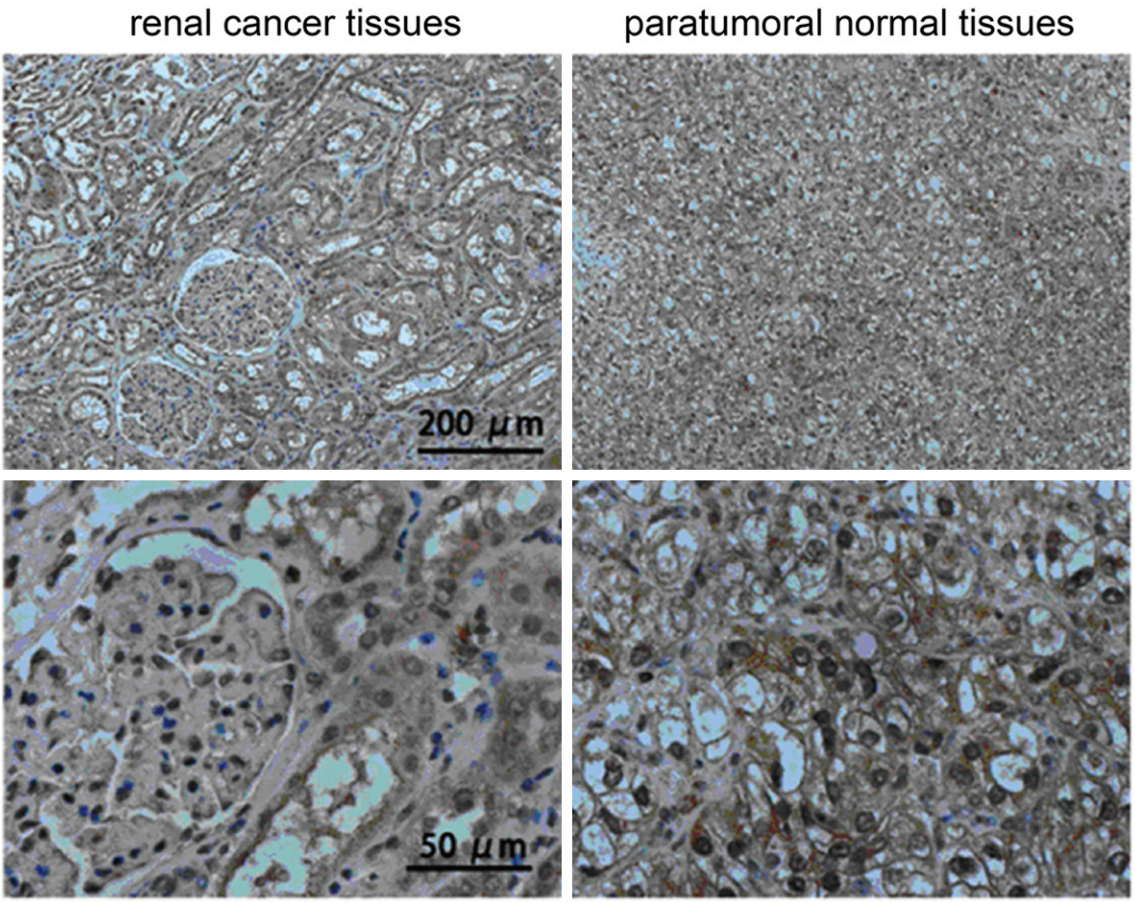

Figure 3. Typical immunohistochemistry staining results of RAGE expression in renal cancer tissues and paratumoral normal tissues.

nation. All of the patients had complete clinical pathological data and signed informed consent before surgery. Patients with trauma or systemic disorders were excluded, and follow-up investigations on the participants were performed for 3-60 months.

Immunohistochemistry and HE staining

Immunohistochemistry staining was performed according to a routine method. Surgically resected tissue specimens were immediately immersed in neutral formalin, maintained at 4°C for one day, and then embedded in paraffin. The embedded tissues were sectioned into 5-m tissue sections using a microtome. The tissue sections were dewaxed with xylene and rehydrated through a series of ethanol gradients, and then, antigen retrieval and blocking was performed with rabbit immunosera. The sections were then incubated with antibodies against HMGB1 and RAGE (Abcam, US) at 37°C for 2 hours. After a thorough washing with phosphate buffered saline (PBS) on a shaker, the tissue sections were incubated with a secondary antibody for 1 hour, washed with PBS, and then incubated with horseradish peroxidase-conjugated streptavidin for 15 minutes. After washing with PBS, the tissue sections were developed with 3, 3'-diaminobenzidine-tetrahydrochloride (DAB), stained with hematoxylin, and observed under a microscope. Sections that were processed with PBS instead of the primary antibody were used as the negative control.

The immunohistochemistry results were determined according to the standards published in the literature [4]. Positive staining of HMGB1 was determined based on the presence of brown stains in the cytoplasm or nucleus, whereas positive staining of RAGE was determined based on the presence of brown stains in the cell membrane. The results were reviewed by two experienced pathologists in a double-

Table 1. Analysis of HMGB1 and RAGE expression in renal cancer tissues and paratumoral normal tissues

Group	Total number of cases	HMGB1		*P*	RAGE		*P*
		Positive cases (%)	Negative cases (%)		Positive cases (%)	Negative cases (%)	
Cancer tissue	80	71 (88.75)	9 (11.25)	< 0.001	72 (90)	8 (10)	< 0.001
Paratumoral normal tissue	80	25 (31.25)	55 (68.75)		27 (33.75)	53 (66.25)	

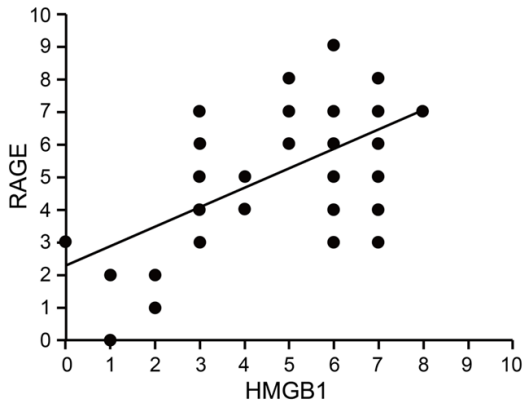

Figure 4. Correlation analysis of expression levels of HMGB1 and RAGE proteins.

blinded evaluation. Fifteen fields were selected on each slide and observed under a microscope (×400). The positive-cell rates were calculated as the average number of HMGB1 or RAGE-positive cells in 100 cells, and the average from the 15 fields was used as the final result for each pathological slide. Four slides were prepared for each patient, and the average positive-cell rate of the 4 slides was used as the value for each patient. The immunohistochemistry results were determined according to the literature and primarily based on two aspects: percentage of positive cells (0 points for below 1%, 1 point for 1-10%, 2 points for 10-30%, 3 points for 30-60% and 4 points for greater than 60%) and staining intensity of positive cells (0 points for negative staining, 1 point for light yellow staining, 2 points for yellow staining and 3 points for brown staining). In the final results, 0-2 points was considered negative and 3-7 points was considered positive [5].

Statistical analysis

Statistical analyses were performed using IBM Statistical Package for the Social Sciences (SPSS) 13.0 (Chicago, IL, USA); a χ^2 test was employed for the count data, and the Kaplan-Meier method was used for the survival analysis. The correlation between the expression of RAGE and HMGB1 in renal carcinoma tissues was analyzed using Spearman's rank correlation coefficient, and statistical significance was measured at $P < 0.05$.

Results

Expression of HMGB1 and RAGE proteins in renal cancer tissues and paratumoral normal tissues

All of the tumor tissue specimens were confirmed by postoperative pathology results (see typical pathological changes in **Figure 1**). Positive expression of HMGB1 in renal cancer tissues was manifested as brown staining in the cytoplasm or nuclei (**Figure 2**), whereas positive RAGE protein expression was manifested as brown staining in the cell membrane (**Figure 3**). The statistical analysis indicated an HMGB1-positive rate of 71% in renal cancer tissues and 25% in the paratumoral normal tissues, and the difference was statistically significant (**Table 1**, $P < 0.05$). The RAGE-positive rate was 72% in renal cancer tissues and 27% in the paratumoral normal tissue, which was also statistically significant (**Table 1**, $P < 0.05$). In addition, the expression of HMGB1 protein in renal cancer tissues exhibited a significant positive correlation with the expression of RAGE protein in these tissues (**Figure 4**, $P < 0.05$).

Relationship analysis between the protein expression of HMGB1 and RAGE and clinical pathological characteristics of renal carcinoma

The expression level of HMGB1 was not correlated with tumor size and gender and age of the patients with renal carcinoma (**Table 2**, $P > 0.05$), although it displayed a significant correlation with clinical stage, differentiation degree and lymph node metastasis in the patient (**Table 2**, $P < 0.05$). Similarly, the expression of

Table 2. Relationship between HMGB1/RAGE protein expression and clinical pathological characteristics of renal carcinoma patients

Group	Total number of cases	HMGB1		*P*	RAGE		*P*
		Positive cases	Negative cases		Positive cases	Negative cases	
Gender							
Male	44	41	3	0.2861	42	2	0.131
Female	36	30	6		30	6	
Age (year)							
< 65	58	52	6	0.7005	53	6	0.6979
≥ 65	22	19	3		19	3	
Degree of differentiation							
Medium/Low	49	48	1	0.0018	48	1	0.0047
High	31	23	8		24	7	
Clinical stage							
I/II	28	21	7	0.0075	22	6	0.0195
III/IV	52	50	2		50	2	
Lymph node metastasis							
Yes	42	41	1	0.0115	41	1	0.024
No	38	30	8		31	7	
Tumor size							
< 4 cm	25	20	5	0.1289	21	4	0.2489
≥ 4 cm	55	51	4		51	4	

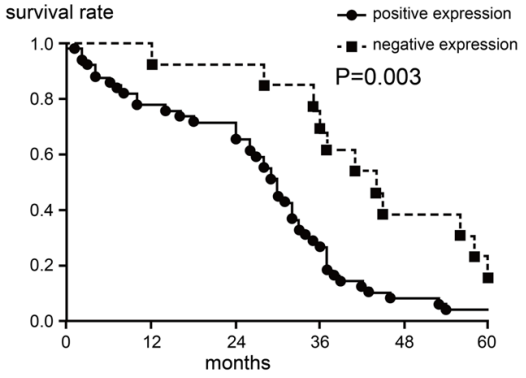

Figure 5. Relationship between HMGB1 protein expression and clinical prognosis of renal carcinoma patients.

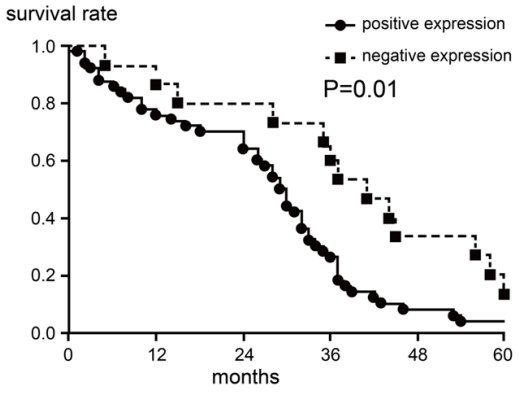

Figure 6. Relationship between RAGE protein expression and clinical prognosis of renal carcinoma patients.

RAGE, the receptor of HMGB1, was not correlated with tumor size and gender and age of the patient ($P > 0.05$), although it had a close relationship with clinical stage, differentiation degree and lymph node metastasis of the renal carcinoma patients (**Table 2**, $P < 0.05$), indicating a consistent relationship between the expression of HMGB1 and RAGE and clinical pathological characteristics of the renal carcinoma patients.

Correlation analysis of the expression of HMGB1 and RAGE proteins and prognosis of renal carcinoma patients

The results show that the 5-year survival rate of the patients with positive HMGB1 and RAGE protein expression was significantly lower than that of the patients with negative HMGB1 and RAGE protein expression (**Figures 5**, **6**, $P < 0.05$).

Discussion

The human HMGB1 gene is located on chromosome 13q12, and it encodes a protein with a molecular weight of approximately 30 kD. HMGB1 is widely distributed in the human body, including in the heart, liver, kidney, and brain tissues. HMGB1 has shown varied cellular localization in different organs, and it is mainly located in the cytoplasm in liver and brain tissue and in the nucleus in most other tissue [6]. HMGB1 participates in the formation of nucleosomes in the nucleus primarily by facilitating the duplication, transcription and repair of genes. In addition, HMGB1 has been shown to stabilize the nucleus and regulate the activity of steroid receptors. HMGB1 also functions as a cytokine when it is translocated from the nucleus to the extracellular space. HMGB1 is actively secreted or diffused into the extracellular space primarily through the following methods: first, certain cells secrete HMGB1 through a mechanism similar to that of autocrine or paracrine in which lysosomal exocytosis is activated by lysophosphatidylcholine; second, HMGB1 is secreted by monocytes-macrophages upon stimulation by proinflammatory cytokines; and third, HMGB1 is passively released into the extracellular space by necrotic or damaged cells [7]. Extracellular HMGB1 must bind to its receptor on the surface of cell membrane to exert its biological effects, and HMGB1 has at least five types of receptors, among which the transmembrane protein RAGE of the immunoglobulin family is a representative receptor. After binding to RAGE, HMGB1 further activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways [8]. In renal carcinoma, elevated NF-κB activity results in the generation of a large number of target gene products (COX-2, matrix metalloproteinase-2 (MMP-2)) and inhibitor of apoptosis protein c-IAP, thereby contributing to tumorigenesis, progression and invasion as an important prognostic factor [9]. Similarly, activation of the MAPK signaling pathway is closely related to the development, progression, invasion and proliferation of renal carcinoma, and inhibition of MAPK activity promotes the apoptosis of renal carcinoma cells [10].

A large number of recent studies have shown that significantly increased expression of HMGB1 occurs in multiple tumor tissues, and its expression in pancreatic cancer and gastric cancer is closely related to tumor development, progression, infiltration and lymph node metastasis, which is likely caused by HMGB1 that is actively secreted into surrounding tissues from tumor cells or passively released by necrotic tumor cells. Secreted or released HMGB1 acts on the membrane receptor of surrounding cells to promote the proliferation and migration of adjacent tumor cells and induce the regeneration of small blood vessels, which facilitates tumor growth and distant migration. Therefore, the inhibition of HMGB1 expression significantly suppressed tumor growth and invasion. Previous studies have found elevated expression of RAGE protein in multiple tumor tissues, and RAGE is known to play an important role in tumor proliferation and invasion [11, 12]. This study showed that the expression levels of HMGB1 and RAGE proteins in renal carcinoma tissues were significantly higher than those in the normal paratumoral tissue. In addition, HMGB1 expression exhibited a significant positive correlation with RAGE protein expression. Moreover, the expression levels of HMGB1 and RAGE were not related to gender, age and tumor size, although they were closely associated with clinical stage, differentiation degree and lymph node metastasis. The 5-year survival rate of patients with HMGB1 and RAGE overexpression was remarkably reduced compared with patients who presented with low expression of these two proteins. The above results indicate that HMGB1 likely has a crucial role in the development, progression and invasion of renal carcinoma and the expression level of HMGB1 is an important factor in the prognosis of patients with renal carcinoma.

In summary, HMGB1 protein and its receptor RAGE exhibit significantly increased expression in renal carcinoma tissues and are closely correlated with clinical pathological characteristics and patient prognosis. HMGB1-targeted drug development has important significance in the treatment of renal carcinoma. Therefore, further in vitro and animal studies are required to validate this target and to provide a solid theoretical basis for HMGB1-targeted clinical treatment of renal carcinoma.

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Disclosure of conflict of interest

None.

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